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EFFECTS OF POLYETHYLENE TEREPHTHALATE ON YEAST ALCOHOL DEHYDROGENASE

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(Received August 18, 1989)

SUMMARY

Yeast alcohol dehydrogenase (alcohol: NAD^+ oxidoreductase, EC 1.1.1.1) was adsorbed onto polyethylene terephthalate, a synthetic polymer. The effects of the polymer on the properties of the enzyme were studied. The specific activity of the bound enzyme on protein basis was only 1.2 per cent of the specific activity of the soluble enzyme. The optimum pH for the catalytic activity was strongly shifted toward acidic direction. The apparent temperature optimum of the bound enzyme was identical with that of the soluble form. The apparent Michaelis constants of the bound enzyme were higher for both ethanol and NAD^+. The conformational stability of the enzyme against heat treatment and urea was decreased as a consequence of adsorption.

INTRODUCTION

The catalytic properties and stability of immobilized enzymes are changed by both the coupling procedure and the polymeric matrix as microenvironment. The effects of the microenvironment are very important. In order to eliminate the disturbing effects of the coupling procedure synthetic polymers with adsorptive capacity could be used modelling microenvironmental influences. For the study of enzyme-polymer interaction a yeast alcohol dehydrogenase-polyethylene terephthalate adsorptive complex was prepared. Alcohol dehydrogenase is a typical redox enzyme composed of four subunits (Kägi and Vallee, 1960). The polyethylene terephthalate beads are porous, hydrophobic adsorbents (Heinrichova and Zliechovcova, 1986). The effects of polyethylene terephthalate on the catalytic properties and stability of yeast alcohol dehydrogenase are presented in this paper.

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MATERIALS AND METHODS

Alcohol dehydrogenase was isolated from baker's yeast according to Racker(1955). The specific activity of the enzyme was 140 units per mg protein. One unit of enzyme activity was defined as the amount of enzyme that catalyses the formation of one μmol of acetaldehyde from ethanol per minute at pH 8.5 and at 25°C. In the preparation 70% of the isolated protein was alcohol dehydrogenase practically in a single molecular form as judged by PAGE.

Polyethylene terephthalate (Sorsilen), a spherical bead support with a pore size of 2-3 cm^3g^{-1} and a specific surface of 80-100 m^2g^{-1}, was prepared via the method of Kubanek and Budin (1976) in the Department of Polymers, Institute of Chemical Technology, Prague, and was obtained from the Institute of Chemistry, Slovak Academy of Sciences, Bratislava, in the frame of cooperation. The carboxyl content is very low (5-15x 10^{-5} equiv/g polymer) therefore the possibility of ionic bonding is negligible (Coupek et al., 1981). All other chemicals were commercial preparations of reagent grade (Reanal Laboratory Chemicals, Budapest, Hungary).

Adsorption of alcohol dehydrogenase. One gram of xerogel was washed with 0.05 M phosphate buffer (pH 6.8) and was suspended in 20 ml 0.05 M phosphate buffer (pH 6.8), and 5 ml enzyme (500-1500 units) was added. The suspension was stirred for 24 hours at 4°C. The gel was next filtered off by suction, and was washed three times with 20 ml 0.05 M phosphate buffer (pH 6.8), three times with the same buffer containing 1 M sodium chloride, and three times with 20 ml 0.1 M triethanolamine/HCl buffer (pH 8.5) to remove the weakly bound proteins. The adsorbed enzyme was stored in 0.1 M triethanolamine buffer (pH 8.5) until use.

Protein determination. Protein determinations were performed according to Lowry et al. (1951). The amount of adsorbed protein was determined indirectly from the difference between the amount of protein introduced into the coupling reaction mixture and the amount of protein present in the filtrate and washing solutions after immobilization.

Assay of alcohol dehydrogenase activity. The activities of both the soluble and the immobilized enzyme were determined spectrophotometrically by following the change in absorbance at 340 nm (Racker, 1950). In the activity assay of the soluble enzyme, the reaction mixture (3 ml) contained 86 mM triethanolamine buffer (pH 8.5), 20 mM ethanol, 1.8 mM NAD^+ and 72.8 mM semicarbazide. The reaction was initiated by the addition of 20-50 μl alcohol dehydrogenase (0.5-1.0 unit). The reaction rate was calculated from the formation of NADH at 25°C, in the first 15-30 seconds after starting the reaction.

In the case of immobilized alcohol dehydrogenase, 50-100 mg of the immobilized enzyme (loaded polymer beads) was suspended in 5 ml 0.1 M triethanolamine/HCl buffer (pH 8.5) containing ethanol, NAD^+ and semicarbazide in the same concentrations as given for the activity test of the soluble enzyme, and the suspension was stirred for an appropriate time

(1-10 min) at 25°C. The enzyme was then filtered off quickly (a few seconds) and the amount of NADH in the filtrate was determined at 340 nm. The product formation was linear with the time until 30 min at least.

Stability tests. Stability tests were performed in 0.05 M potassium phosphate buffer (pH 6.8). The inactivation was monitored via assaying of the residual enzyme activity at appropriate time intervals under standard conditions.

RESULTS

Catalytic activity

The highest bound enzyme activity was achieved when the process was carried out in 0.05 M phosphate buffer (pH 6.8), with about 600 units enzyme and 4 hours for the adsorption at 4°C. In this case, the bound enzyme activity was 27-35 units per gram xerogel. The specific activity of the bound enzyme was about 1.7 unit per mg protein, i.e. 1.2% of that of the native enzyme. The protein leakage was only 3.3% during 4 days of storage in 0.1 M triethanolamine buffer (pH 8.5) at 8°C.

pH-dependence of catalytic activity

The pH-dependence of the catalytic activity was studied in 0.1 M glycine/NaOH buffer in the pH range 7.5-12.0. The optimum pH for the catalytic activity of the bound enzyme was about 8.5 (Fig. 1).

Temperature-dependence of activity

The temperature-dependence of the activity of the soluble and of the bound alcohol dehydrogenase was determined in triethanolamine buffer (pH 8.5) in the temperature range 25-50°C. The reaction time was 5 minutes. The apparent temperature optimum for both the soluble and the immobilized enzyme was about 30°C (Fig. 2).

Michaelis constants

The effect of the substrate concentration on the rate of the alcohol dehydrogenase-catalysed reaction was studied in the concentration range 1-20 mM for ethanol and 0.1-1.5 mM for NAD^+ in triethanolamine/HCl buffer (pH 8.5) at 25°C. Lineweaver-Burk plots were used to determine the Michaelis constants (K_m). The apparent K_m value of the immobilized alcohol dehydrogenase with ethanol as substrate was 92.59 mM, and was

higher than that of the soluble enzyme (11.9 mM). The $K_{m\ app}$ value of the immobilized enzyme for NAD^+ substrate was 0.66 mM, while that of the soluble form was 0.19 mM.

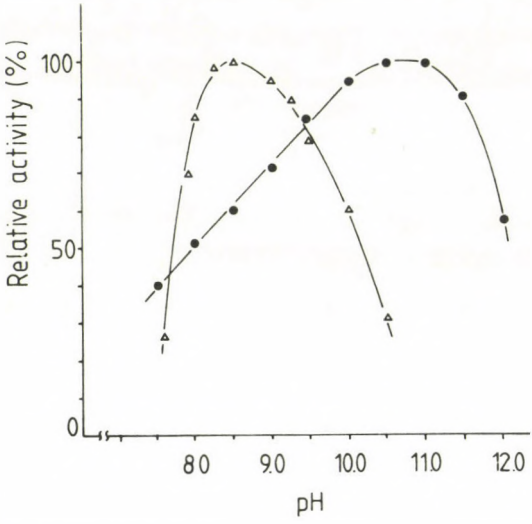

Fig. 1. Effect of pH on the activity of native (●) and bound (△) alcohol dehydrogenase. Experiments were carried out in 0.1 M glycine/NaOH buffer at 25°C, with ethanol as substrate. For both the native and the bound enzyme, the maximum activity was taken as 100%.

Thermal stability

The heat inactivation of alcohol dehydrogenase was studied in 0.1 M phosphate buffer (pH 6.5) at 45°C. The inactivation of the bound enzyme was very fast compared with that of the soluble one. After incubation for 30 minutes, the bound enzyme had, practically lost all of its activity while the soluble one still preserved about 80% of its activity (Fig. 3).

Stability against urea

The effect of urea on the conformational stability of alcohol dehydrogenase was studied at 3 and 4 M urea in 0.1 M phosphate buffer (pH 6.5) at 25°C. At both urea concentrations, the soluble enzyme was inactivated more slowly than the bound enzyme (Fig. 4).

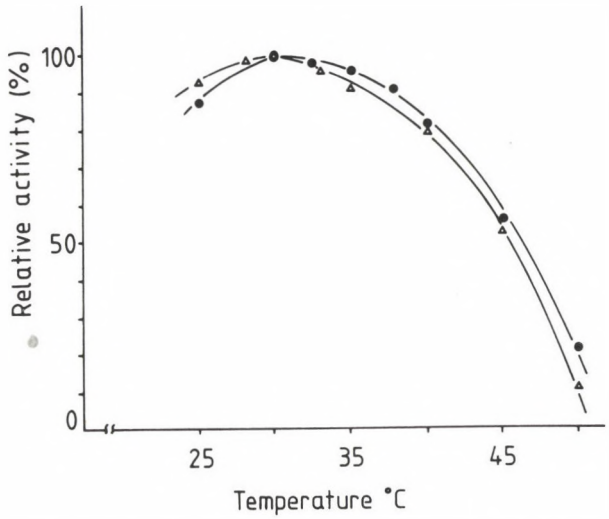

Fig. 2. Effect of temperature on the activity of native (●) and bound (Δ) alcohol dehydrogenase. Experiments were carried out in 0.1 M triethanolamine buffer (pH 8.5). The maximum activities (soluble enzyme: 5.6 units ml^{-1}, immobilized enzyme: 1.6 units ml^{-1}) were taken as 100%.

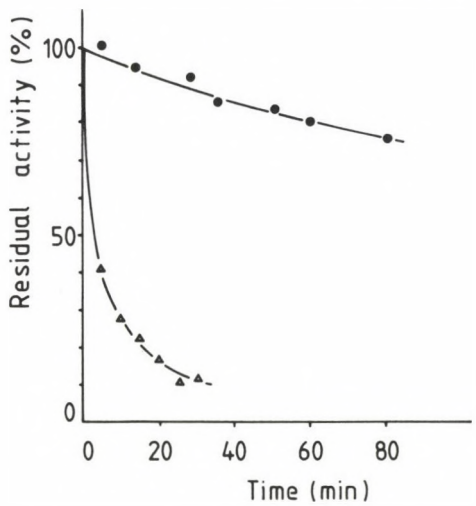

Fig. 3. Thermal inactivation of native (●) and bound (Δ) alcohol dehydrogenase at 45°C. Experiments were carried out in 0.1 M phosphate buffer (pH 6.5). The starting activities were taken as 100%.

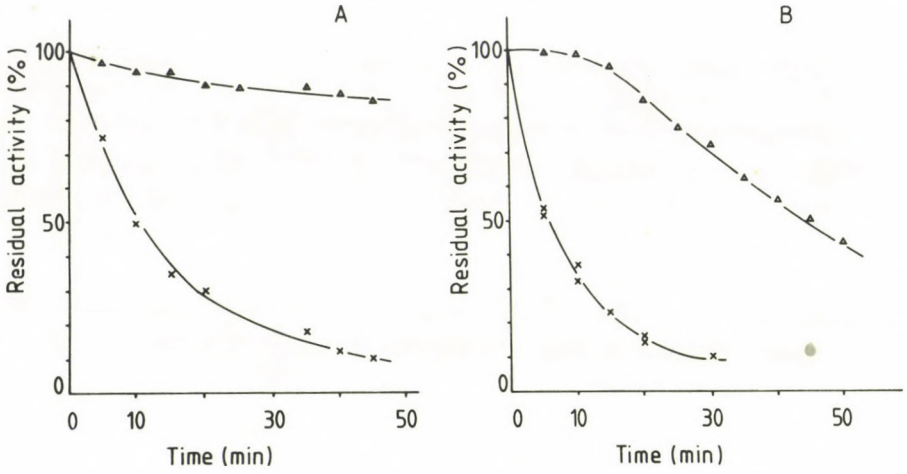

Fig. 4. Effect of 3 M (Δ) or 4 M (x) urea on the activity of soluble (A) and bound (B) alcohol dehydrogenase. The starting activities were taken as 100%.

DISCUSSION

The investigation of enzyme-polymer interactions could reveal some changes in the catalytic properties and conformational stability of immobilized enzymes caused by the polymeric support as microenvironment. Some data refer to it that the hydrophobic matrices damage the conformational stability of immobilized enzymes (cf. Zaborsky, 1974). Testing the problem, yeast alcohol dehydrogenase was adsorbed on the surface of polyethylene terephthalate, a porous hydrophobic adsorbent. The adsorption of alcohol dehydrogenase was strong, the protein leakage was only 3.3% during 4 days of storage. The adsorption led to a marked inactivation of the enzyme. The causes of the inactivation might be a conformational change in the tertiary structure, or steric hindrance of the active sites by a structural moiety of the polymer, or both together. The catalytic properties of the bound enzyme are similar to those of the native enzyme, except for the $K_{m\ app}$ values, which were higher than those of the native enzyme for both the substrate and the coenzyme. The alteration in the $K_{m\ app}$ values might be a consequence of a complex interaction between

the polymer matrix and the enzyme molecules and/or conformational changes caused by the adsorption.

The adsorption on the hydrophobic polymer surface is disadvantageous for the conformational stability of alcohol dehydrogenase, as indicated by the results of heat inactivation and urea treatment.

The high degree of inactivation and the decrease in the conformational stability emphasize that the hydrophobic matrices are unsuited for the enzyme immobilization.

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FACTORS INFLUENCING THE OPERATION OF A VERTICAL BIOREACTOR SEGMENTED WITH PERFORATED PLATES

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SUMMARY

Factors influencing the operation of a vertical bioreactor segmented with perforated plates supporting immobilized yeast cells were studied. It was found that the most important factors are the length-diameter (L/D) ratio of the reactor and the dilution rate. It was supposed that the optimal L/D ratio is about 1. The operation of the reactor was more favourable at a low liquid phase-solid phase ratio. The spatial distribution of the biocatalyst had only a slight, if any effect. The periodically changed direction of feed flow has no improving effect on the fermentation process.

INTRODUCTION

The continuous production of ethanol using immobilized microbial cells is a recent progress in applied sciences. Successful pilot plant and industrial operations are known (Nagashima et al., 1983, 1984; Najima et al., 1987). Different vertical packed-bed and fluidized bed reactors are preferentially used (Godia et al., 1987). In our work for laboratory and pilot plant purposes a simple bioreactor segmented with perforated plates was constructed. Some factors influencing the operation of the bioreactor were studied.

MATERIALS AND METHODS

Chemicals. Yeast extracts and peptones were obtained from Merck AG (Darmstadt, FRG), Porapak Q was purchased from Serva Feinbiochemica GmbH (Heidelberg, FRG) and sodium alginate of Protanal SF 120 type from Protan and Fragertun A.S. (Drammen, Norway). All other chemicals were reagent grade or of the commercially available highest purity from Reanal Factory of Laboratory Chemicals (Budapest, Hungary).

Microorganism. Saccharomyces cerevisiae SCI strain was obtained from the Research Institute of Alcohol Industry (Budapest, Hungary).

Culture medium. One litre of the medium contained: glucose; 10 g; peptone, 5 g; yeast extract, 3 g and malt extract, 3 g. The pH was adjusted to 5.0 and the medium was sterilized at 120°C for 20 minutes.

Fermentation medium. One litre of the medium contained: glucose, 100 g; yeast extract, 1.5 g; NH_4Cl, 2.5 g; K_2HPO_4, 5.5g; $MgSO_4 \cdot 7H_2O$, 0.25 g and $CaCl_2$. 5 g. The medium was sterilized at 120°C for 20 minutes. The pH was 4.1 (Wada et al., 1979).

Growth conditions and preparation of cell suspensions. Cells were cultivated at 30°C in sterile growth medium for 24 h in a controlled environment incubation shaker (Model R76, New Brunswick Co., Edison N.J., USA) at 128 rpm. After incubation the viable cell number was 3.5×10^7 cells ml^{-1}. This cell suspension was used for the experiments.

Immobilization of yeast cells. The yeast cell suspension was immobilized by homogeneous suspension in a 2.5% sodium alginate gel solution (Kierstan and Bucke, 1977). The cell density was $1\text{-}5 \times 10^6$ cells ml^{-1}. Beads were formed by dripping the suspension through a syringe into a sterile 1% calcium chloride solution. The average bead diameter was 4 mm. The immobilized yeast cells were grown for 24 h at 30°C in a shaker at a gel/culture medium ratio 1:2 (w/v).

Determination of cell number. The cell number was evaluated after the dissolution of gel beads. Two gel particles were placed into a test tube containing a 1 ml solution composed of 3% sodium chloride and 2% EDTA and were gently shaken at room temperature for dissolving. The total cell number was counted using a Burker Chamber. For the determination of the number of living cells, decimal dilutions in physiological saline were plated on agar containing yeast extract (0.3%), peptone (0.5%), glucose (1.0%) and malt extract (0.3%). Counts were performed after incubation at 30°C for 48 h.

Analytical methods. The ethanol concentration was determined using a Chrom 4 type GC chromatograph (Laboratorni pristroje, Prague, Czechoslovakia) equipped with a flame ionization detector and a Porapak Q (80-100 mesh) column (250 cm long and 3 mm i.d.). Nitrogen was used as the carrier gas and methanol as an internal standard. Glucose concentrations were determined using a glucose oxidase enzyme electrode (OP-GL-7113-S type, Radelkis Electrochemical Instruments, Budapest, Hungary).

RESULTS

Bioreactors

Bioreactors were constructed by assembling glass column elements with 47 or 100 mm internal diameters. The inner spaces of the reactors were equally divided by perforated

trays supporting the biocatalyst (Fig 1). Sterilized feed media were pumped from a reservoir into the bottom or to the top of the columns. Samples were withdrawn from effluents and from each segment of the reactors and assayed for ethanol and glucose concentration.

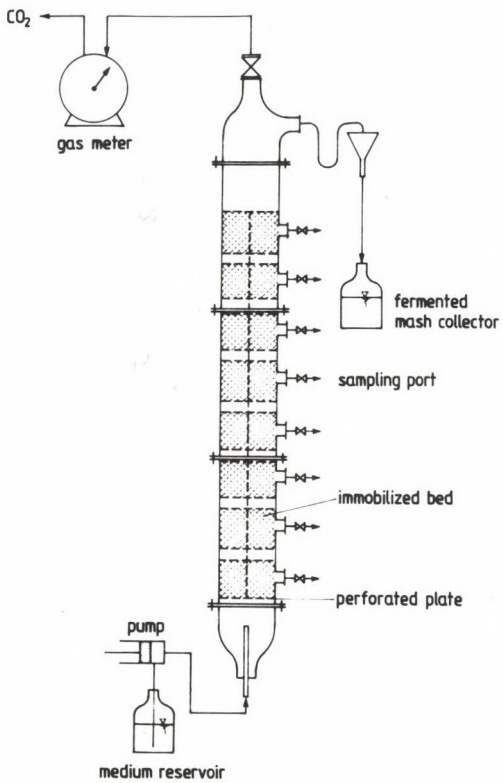

Fig. 1. Vertical bioreactor segmented with perforated plates

Effect of liquid phase-solid phase ratio on the fermentation process

Reactors (520 ml each) were assembled with a length/diameter (L/D) ratio of 6.4. Into the reactors 50, 100 and 200 g of alginate beads containing yeast cells ($9x10^7$ cell ml gel^{-1}) were packed, respectively. A dilution rate of 0.13 h^{-1} was applied. The performance of reactors was compared once steady

state had been reached (3-7 days). The operation of the reactors were more favourable at a low liquid phase-solid phase ratio. The volumetric productivity increased with the weight of the biocatalyst (Table 1).

Table 1. Effect of liquid phase-solid phase ratio on the fermentation process

	Wet weight of immobilized biocatalyst (g)		
	50	100	200
Liquid-solid ratio			
($ml \cdot g^{-1}$)	9.6	4.4	1.8
($ml \cdot ml^{-1}$)	12	5.3	2.2
Ethanol concentration in the effluent			
($g \cdot l^{-1}$)	33	38	47
Residual glucose concentration in the effluent			
($g \cdot l^{-1}$)	32	24	6
Volumetric productivity for ethanol relating to the total volume			
($g \cdot l^{-1} \cdot h^{-1}$)	4.3	4.9	6.1

Effect of spatial distribution of the biocatalyst

Reactors (760 ml each) with L/D ratio of 9.3 containing 250 g wet biocatalyst ($1x10^8$ cell ml gel^{-1}) were used at a dilution rate of 0.11 h^{-1}. The number of perforated trays supporting the biocatalyst was varied. The ethanol concentration and productivity was only slightly influenced by the spatial distribution of biocatalyst (Table 2).

Table 2. Effect of spatial distribution of the biocatalyst

	Number of trays in the reactor				
	1	3	6	11	21
Ethanol concentration in the effluent ($g \cdot l^{-1}$)	41	41	43	44	47
Residual glucose concentration in the effluent ($g \cdot l^{-1}$)	20	20	15	13	6
Volumetric productivity for ethanol relating to the total volume ($g \cdot l^{-1} h^{-1}$)	4.5	4.5	4.7	4.8	5.2

Effect of L/D ratio of the bioreactors

The effect L/D ratio of bioreactors on the productivity was studied in reactors assembled with different L/D ratios. The productivity of the bioreactors rapidly increased when the L/D ratio decreased below 2. It was supposed that the optimal L/D ratio would be about 1 (Fig. 2).

Ethanol production at different dilution rates

To study the effect of dilution rate on the ethanol production a reactor was assembled with an L/D ratio of 0.85 and a liquid phase-solid phase ratio of 2.0. The biomass concentration was 1.6×10^8 cell ml gel^{-1}. Comparisons were made at steady state conditions.

At the given range between 0.09 and 0.94 h^{-1} ethanol productivities increased as the dilution rates were increased (Fig. 3).

Effect of feed flow direction on the fermentation process

Substrate (glucose) and product (ethanol) gradients developing along the length of the reactor disadvantageously

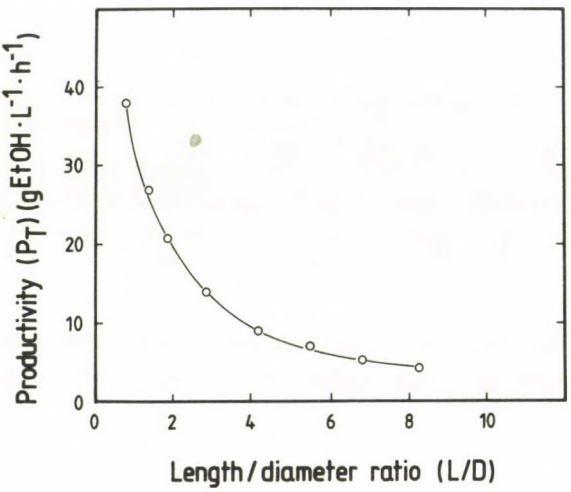

Fig. 2. Ethanol production at different L/D ratios of bioreactors

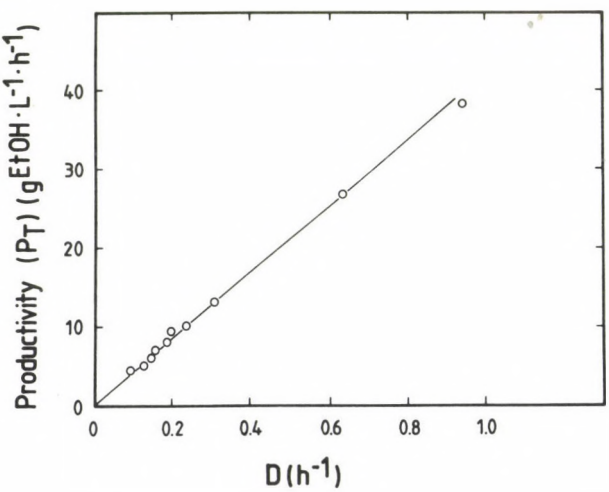

Fig. 3. Ethanol production at different dilution rates

influence the fermentation process. For the elimination of gradients the direction of feed flow was periodically changed. The steady state glucose and ethanol concentration profiles along the length of reactor are presented in Fig. 4 representing five cycles.

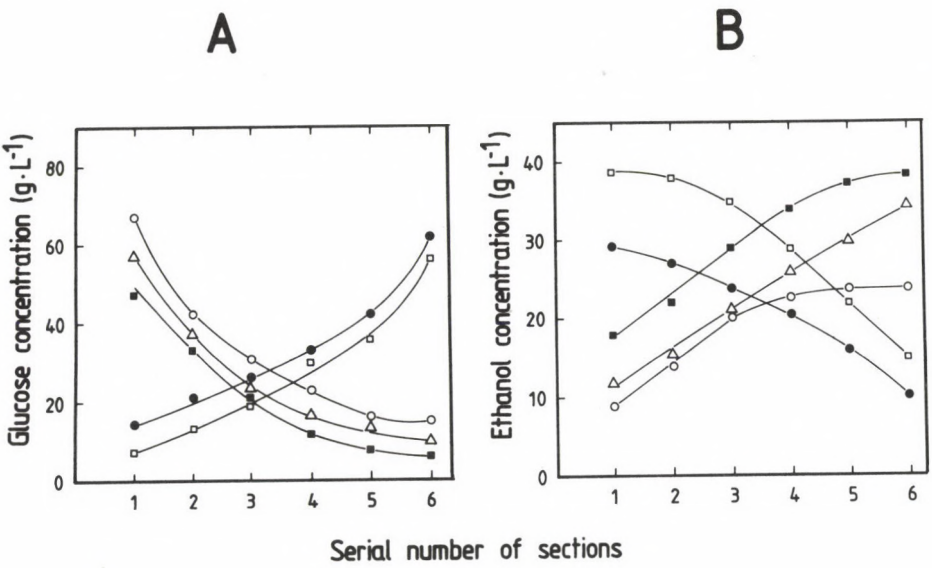

Fig. 4. Effect of feed flow direction on the fermentation process. (A) Glucose consumption; (B) Ethanol production. (o) 1st cycle (upflow); (●) 2nd cycle (downflow); (Δ) 3rd cycle (upflow); (□) 4th cycle (downflow); (■) 5th cycle (upflow).

It was found that the yeast cells could not be adapted to the often changing conditions. After 35 days operation the number of living cells was decreased (total cell number, 9.4×10^8 cell ml gel^{-1}; living cells, 4.5×10^6 cell ml gel^{-1}), while in the control experiment without changing the flow direction the living cell number was 4.3×10^7 ml gel^{-1}.

DISCUSSION

A simple but effective vertical bioreactor was constructed for the continuous production of ethanol from mono

and oligosaccharides. The bioreactor was segmented with perforated plates supporting immobilized yeast cells. The plates help to eliminate the canalization disturbing the optimal effect of biocatalyst beads. Some of the factors influencing the operation were studied. It was found that the most important factors are the length-diameter (L/D) ratio of the reactor and the dilution rate. The effect of dilution rate in the bioconversions has been well-known. It was supposed that the optimal L/D ratio would be about 1. Similar "dumpy" fluidized bed reactors with and L/D ratio of 1.5-2 were used for pilot plant operation (Nagashima et al., 1983, 1984). The operation of the reactor was more favourable at a low liquid phase-solid phase ratio. The spatial distribution of the biocatalyst had only a slight, if any effect.

The applicability of bioreactor was judged up to 100 l scale with sugar-beet molasses in the fermentation medium.

Acknowledgements

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THE EFFECT OF MEMBRANE POTENTIAL ON THE LIMITED TRYPTIC DIGESTION OF THE SARCOPLASMIC RETICULUM Ca^{2+}-ATPase

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SUMMARY

The tryptic cleavage process of the sarcoplasmic reticulum Ca^{2+}-ATPase was analysed under the influence of experimentally generated membrane potential. The digestion of the Ca^{2+} transport enzyme was stopped before the dissipation of the potential response. The cleavage products reflected the actual conformation of the enzyme as the low Ca^{2+} affinity E_2, under the influence of inside positive, and as the high Ca^{2+} affinity E_1 conformation under the influence of inside negative potential. These results provide further support for the possible role of transient membrane potential changes in the regulation of the conformational equilibrium of the sarcoplasmic reticulum Ca^{2+} pump enzyme.

INTRODUCTION

Each reaction cycle of the sarcoplasmic reticulum Ca^{2+}-ATPase carries two Ca^{2+} atoms into the SR lumen on the expense of one ATP molecule (1). The amount of the transported Ca^{2+} would be enough for the generation of a membrane potential of about 100 mV (2). X-ray microanalysis studies did not reveal Cl or K gradients accross the SR membrane in frozen sections of resting and fatigued muscles (3). Several monovalent ion channels in the SR membrane can eliminate the potential changes associated with the Ca^{2+} translocation process (2).

SR membrane preparations are suitable for the generation of transient membrane potential by ion exchange-dilution methods. Potential sensitive fluorescent dyes demonstrate the existence of these potential responses for about 1-2 minutes after the dilution (4).

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Arteficially generated inside negative membrane potential stimulated the Ca^{2+} transport and Ca^{2+} dependent ATPase activity in SR preparations. Inside positive potential had the opposite effect on these functions (4). Inside positive potential strongly accelerated the vanadate induced two-dimensional crystal formation of the enzyme, meanwhile inside negative potential quickly destroyed the previously formed enzyme arrays (5, 6). These results, together with the fluorescence signals of the Ca^{2+}-ATPase enzyme supported the conformation specific effects of the membrane potential in the SR (7).

The tryptic cleavage process of the SR Ca^{2+}-ATPase is sensitive to the actual conformation of the enzyme. The first cleavage at Arg 505, takes place in both main conformational states, meanwhile the second cleavage site, at Arg 198, is completely protected from the tryptic digestion in the low Ca^{2+} affinity, E_2 conformation (6, 8).

In the present work we characterized the conformation specific effects of the membrane potential, by following the limited tryptic proteolysis of the sarcoplasmic reticulum Ca^{2+}-ATPase.

MATERIALS AND METHODS

Sarcoplasmic reticulum vesicles were prepared from the fast-glycolytic back muscles of rabbits, by the method of Nakamura et al. (9). The final microsomal pellet was suspended in 0.15 M K-glutamate, 10 mM Imidazole, pH 7.4 (KG-samples) or in 0.15 M Choline-Cl, 10 mM Imidazole, pH 7.4 (CC-samples), at 30 mg/ml protein concentration each. The protein concentration was measured with the Folin method, using BSA as standard.

Inside positive potential was generated by diluting the CC-samples in 0.15 M K-glutamate, 10 mM Imidazol, pH 7.4 medium (KG-medium). Inside negative potential was achieved by the dilution of the KG-microsome samples in 0.15 mM Choline-Cl, 10 mM Imidazole, pH 7.4 medium (CC-medium). Dilution of CC-samples in CC-medium as well as KG-samples in KG-medium served as potential free controls. The generation and dissipation of the membrane potential was followed by the absorbance changes of Oxonol VI. dye at 625 and 586 nanometers as described by Beeler et al. (4).

The effect of the membrane potential on the Ca^{2+}-ATPase crystal formation was controlled with negative staining electron microscopy with a JEOL 100 B electronmicroscope at 80 kV accelerating voltage, 15 seconds after the 20 fold dilution

of the CC microsomes in KG-medium supplemented with 5.0 mM Na_3VO_4, 5.0 mM $MgCl_2$ and 0.5 mM EGTA. As control, CC-microsomes were diluted in CC-medium containing vanadate, EGTA and $MgCl_2$.

The tryptic cleavage experiments were carried out at room temperature. The digestion was stopped by the addition of 2x amount of soybean trypsin inhibitor, or 100 µl of SDS-sample buffer (20 mM Tris-Cl, pH 8.0, 10% SDS, 2% β-mercaptoethanol, 20% glycerol, 0.1% Bromphenolblue). The trypsin was added to the samples together with the dilution media, used for the generation of the membrane potential (as described above). The appropriate trypsin-protein ratio as well as the proper digestion time was determined in preliminary experiments. The digestion products were characterized after separation on 6-18% gradient polyacrylamide gels.

RESULTS

The tryptic cleavage products of the SR Ca^{2+}-ATPase suspended in KG and in CC-medium were identical with the ones observed in standard SR preparations suspended in other buffer solutions.

The absorbance change of the Oxonol VI. dye indicated the generation of an inside positive membrane potential upon the dilution of CC-microsomes in KG-medium. The potential signal was fully dissipated 45 seconds after the dilution. The dilution of KG-microsomes into KG-medium did not yielded this kind of absorbance change (Fig. 1).

Rapid two-dimensional crystal formation was clearly visible on the sufrace of CC-microsomes 15 seconds after the dilution in 0.15 M K-glutamate, 10 mM Imidazole, pH 7.4, 5.0 mM Na_3VO_4, 5.0 mM $MgCl_2$, 0.5 mM EGTA solution (Fig. 2a). CC-microsomes diluted in vanadate and EGTA supplemented CC-medium did not show any sign of two-dimensional crystal formation within this period of time (Fig. 2b). On the basis of these observations for the further experiments 15 sec was selected as appropriate digestion time.

During 15 sec digestion a trypsin:protein ratio of 1:40 gave clear appearance of the secondary cleavage products (A_1 and A_2 subfragments, with molecular mass of 32 and 25 kD respectively) of the SR Ca^{2+}-ATPase (Fig. 3). This trypsin-protein ratio was selected for the experiments testing the effects of the membrane potential.

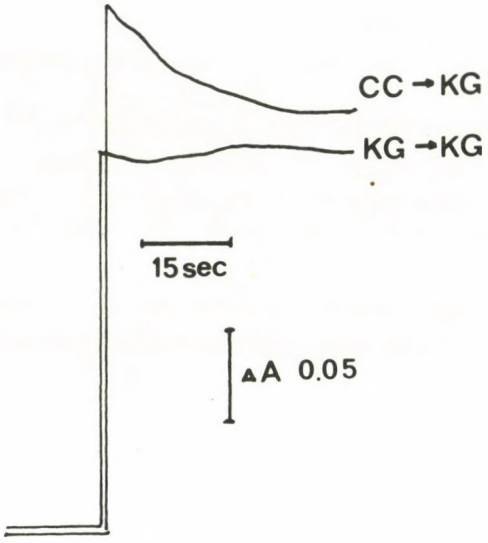


Fig. 1. Absorbance changes of Oxonol VI. dye, upon the dilution of SR vesicles, suspended in Choline-Cl medium into K-glutamate medium (CC-KG), and K-glutamate medium into K-glutamate medium (KG-KG) measured at 625 nm wavelength.

The tryptic cleavage of CC microsomes diluted in KG-medium yielded clearly less A_1 and A_2 subfragments within 15 seconds digestion time, than the KG microsomes did, after the dilution in CC-medium (Fig. 4a). The control samples (CC diluted in CC and KG diluted in KG) contained similar amounts of A_1 and A_2 subfragments to the samples, digested under the influence of inside negative potential (KG microsomes diluted in CC-medium; Fig. 4a, lanes a, d and c).

In the presence of 0.5 mM EGTA the digestion of the enzyme at the second cleavage site was clearly slower in the samples digested without potential generation (Fig. 4b, lanes a and d). The KG microsomes after dilution in CC-medium (inside potential generation) gave more A_1 and A_2 subfragments than the CC microsomes, when diluted in KG-medium (inside positive potential generation), in the presence of 0.5 mM EGTA as well (Fig. 4b, lanes b and c).

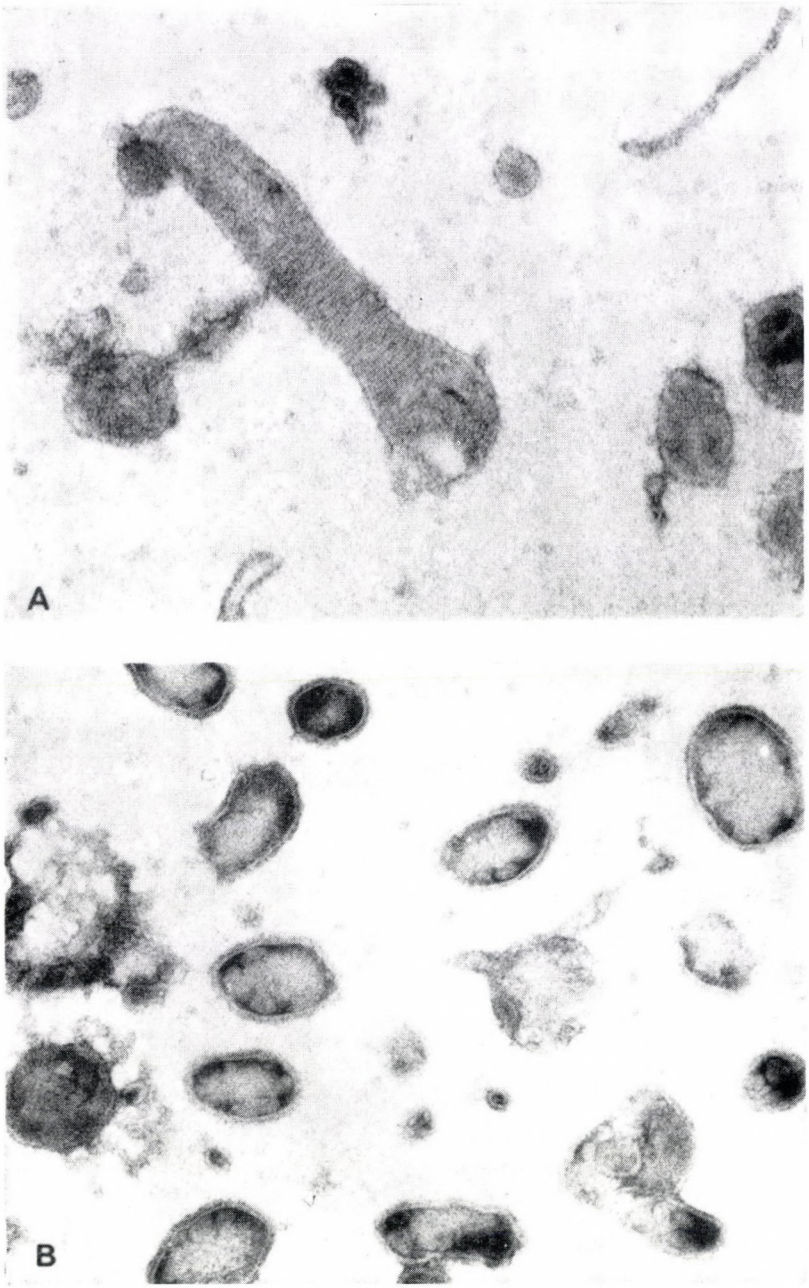


Fig. 2. Negative stained electron micrographs of Choline-Cl medium suspended SR vesicles, 15 seconds after the dilution in 5.0 mM Na_3VO_4, 5.0 mM $MgCl_2$, 0.5 mM EGTA containing K-glutamate medium (A) and Choline-Cl medium (B). Negative staining with 1% uranyl acetate. Magnification 100,000 ×

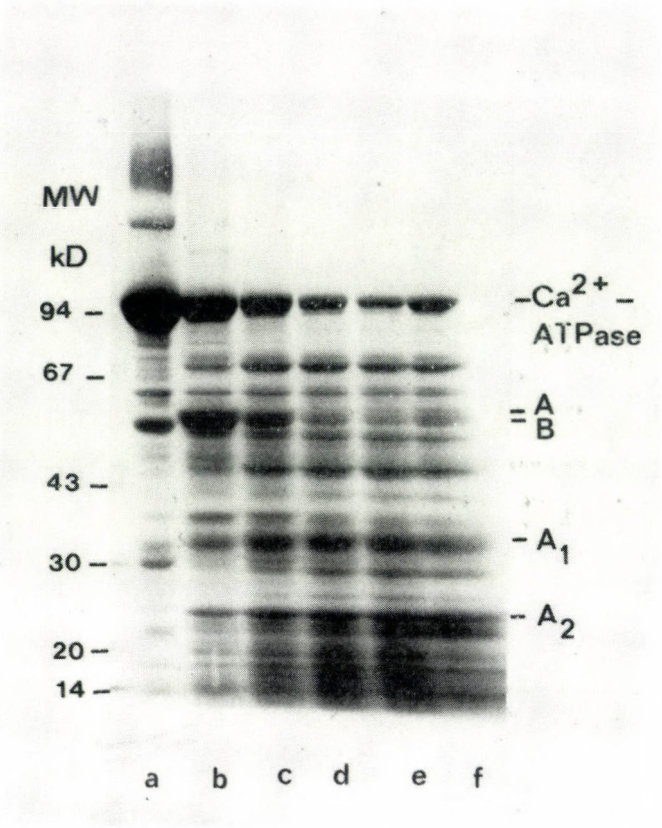
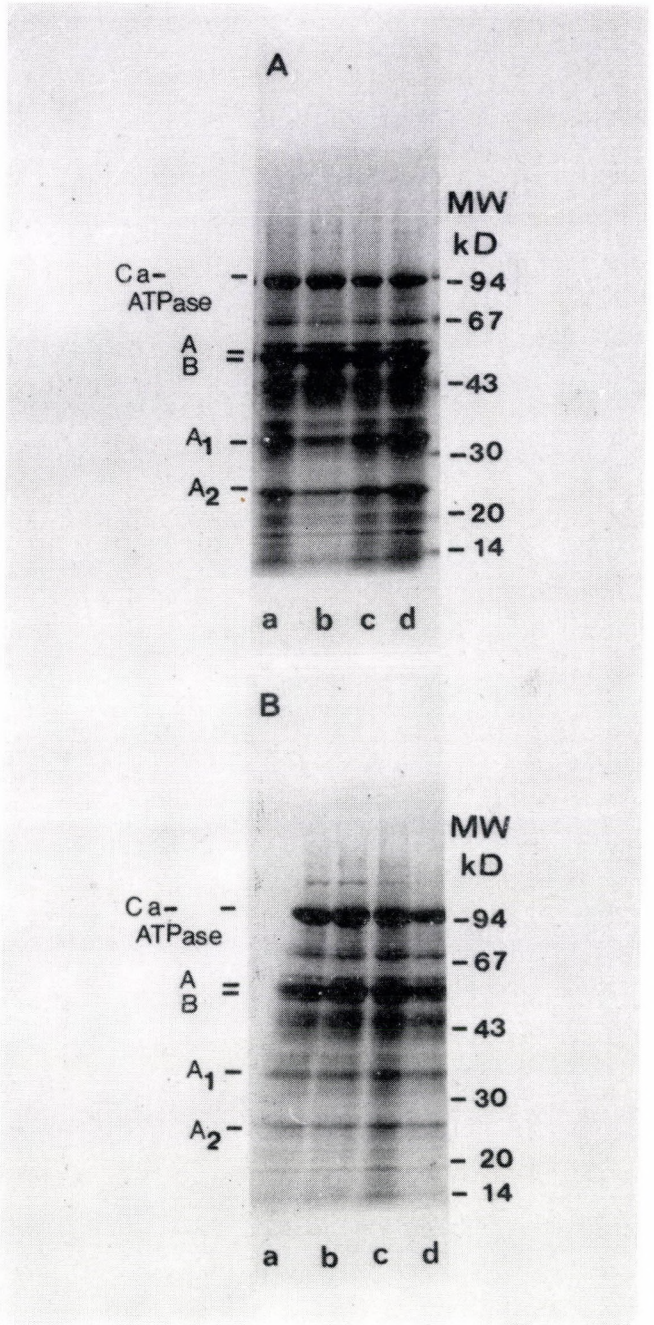


Fig. 3. Peptide composition of sarcoplasmic reticulum preparations after 15 sec tryptic digestion at room temperature. Trypsin : protein ratios: lane a: no trypsin was added, b: 1 : 80, c: 1 : 40, d: 1 : 20, e: 1 : 10, f: 1 : 5. 6–18% gradient polyacrylamide — SDS-gel

Fig. 4. Peptide composition of sarcoplasmic reticulum preparations digested for 15 seconds at room temperature at a trypsin : protein ratio of 1 : 40 in EGTA free medium (panel A), and in the presence of 0.5 mM EGTA (panel B). Lane a: microsomes suspended in CC-medium, and diluted 20 fold in CC-medium (control), b: microsomes suspended in CC-medium and diluted 20 fold in KG-medium (inside positive potential generation), lane c: microsomes suspended in KG-medium and diluted 20 fold in CC-medium (inside negative potential generation), lane d: microsomes suspended in KG-medium and diluted 20 fold in KG--medium (control). The trypsin was added together with the dilution medium. 6–18% gradient SDS-polyacrylamide gels


A
MW
kD
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DISCUSSION

Dilution of SR vesicles, suspended in Choline-Cl medium, into K-glutamate medium generates a trasient inside positive membrane potential. Dilution under the opposite circumstances yields a transient inside negative potential signal (4). Fluorescence or absorbance changes of several voltage sensitive dyes indicate the existence of this potential even 30-60 seconds after the dilution (4, 10).

In the present work the existence period of the membrane potential was used to study the actual conformation of the SR Ca^{2+}-ATPase due to the tryptic cleavage pattern of the enzyme. The digestion was started by adding the trypsin together with the diluting media (generation of the potential), and it was stopped before the dissipation of the potential change. The E_1 conformation is reflected in the cleavage process by the presence of the subfragments A_1 and A_2. In the low Ca^{2+} affinity, E_2 conformation these cleavage products remain absent (8, 11, 12).

The tryptic digestion of the SR Ca^{2+}-ATPase yielded clearly less A_1 and A_2 subfragments under the influence of inside positive potential than under inside negative potential did. The presence of 0.5 mM EGTA in the digestion medium did not alter this difference.

The tryptic cleavage products of the control samples, digested without potential generation were similar to the ones observed under the influence of inside negative potential, when the digestion process was carried out in EGTA free medium. Addition of 0.5 mM EGTA to the digestion medium caused a digestion pattern in the control samples similar to the one, observed under the influence of inside positive potential.

The present results give further evidence for the conformation specific effects of membrane potential on the SR Ca^{2+}-ATPase (5). The inside positive potential seems to induce and stabilize the E_2, the inside negative potential the E_1 conformation. The potential free control samples showed a cleavage pattern corresponding to the E_1 conformation in EGTA free medium. In the presence of EGTA the conformation of the enzyme was retained in the E_2 state (11, 12).

The protection of the second tryptic cleavage site under the influence of inside positive potential was not as complete as it was observed previously in the presence of vanadate and EGTA (6). The SR preparations always contain a certain amount leaky, damaged vesicles, vesicle fragments, which are excluded from the membrane potential generation. The trypsin could freely digest their Ca^{2+}-ATPase content, yielding A_1 and A_2 subfragments on the gels. In the presence of vanadate even the completely solubilized Ca^{2+}-ATPase molecules are fully protected against the second tryptic cleavage.

On the basis of previous electronmicroscopic and fluorescent spectroscopic observations (5, 6, 7) and the present results, the participation of the transient membrane potential changes in the conformational reorganization of the Ca^{2+}-ATPase during the transport cycle, seems to be a reasonable assumption.

Acknowledgement

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Short paper

POTENTIAL PROMOTER SEQUENCE IN THE NON-TRANSCRIBED SPACER OF THE HUMAN RIBOSOMAL RNA GENE

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The eukaryotic rRNA genes are arranged in tandemly repeated arrays in the nucleolus. A repeating unit is assembled from two parts: one of them encodes for the primary rRNA transcript (45S rRNA in mammals) and the other part, the non-transcribed spacer (NTS) probably does not contain sequences for ribosomal RNAs. The coding region of rRNA genes shows remarkable lenght and sequence conservation, but the length and the structure of the NTS exhibit high diversity in various eukaryotes. The length of the NTS is approximately 4.0 kb and 35.0 kb in Xenopus and in human species, respectively (2).

The transcription of rRNA genes, unlike the transcription of mRNA coding genes, is species specific (3). It means, that for example the human cell machinery cannot transcribe the mouse rDNA and vice versa (4). Up to our present knowledge two or three protein factors are necessary for the efficient transcription of rRNA genes. One of them is the RNA polimerase I, and other factor(s) are responsible for the species-specific transcription (5, 6, 7).

Recently we have reported the cloning of the entire NTS of the human rRNA gene (1). Here we present the DNA sequence of a part of the NTS region. It is composed of homologous sequences to a promoter region of the human rDNA and it may bind human specific transcription initiation factor(s).

Enzymes were bought from GIBCO-BRL GmbH (Eggenstein, F.R.G.) and from Amersham Int. (Amersham, England). They were used according to the suppliers recommendations.

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Alfa-32-P-dATP was supplied by the Isotope Institute Hungarian Academy of Sciences (Budapest, Hungary).

DNA sequencing was made with the M13 sequencing kit of Amersham Int. and DNA computer analysis was performed with Stadden-plus software package of Amersham Int. (Amersham, England).

Recently, we have reported (1) the cloning and restriction mapping of the entire human NTS (Fig. 1A). In the middle of the NTS we have identified a new repetitive DNA region, where a 4.6 kb long sequence motif (the XbaI element) was present in two copies (Fig. 1B). We have suggested, that the repeating unit of the human rRNA gene might begin with the second XbaI element of the spacer, and continues toward downstream regions, including the 18S and 28S rRNA coding regions. It terminates at the end of the first XbaI element of the spacer (Fig. 1C). This way the repeating units of the human rDNA were flanked at both ends by long terminal repeats.

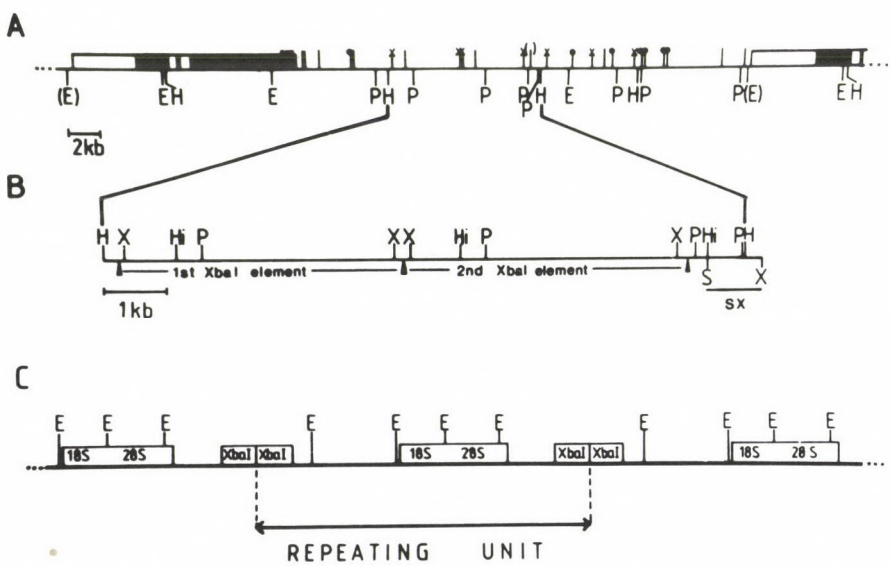

Fig. 1. Structure of the human rRNA gene. A: Restriction map of the NTS of human rRNA gene. The filled bars represent the coding regions for 18S, 5.8S and 28S rRNAs. The open bars show the transcribed spacer sequences. B: Position of the XbaI elements. The sequenced DNA region is underlined and labelled by SX. C: The repeating unit of the human rRNA gene. Abbreviations for restriction enzymes: E-EcoRI, H-HindIII, Hi-HincII, P-PstI, S-SalI and X-XbaI.

In Fig. 2 we present the nucleotide sequence of a SalI-XbaI fragment of the NTS, which allocates close to the 3' flanking sequence of the second XbaI element (SX in Fig. 1B) and joins directly to a formerly reported NTS sequence (XPS in ref. 1).

Noteworthy, a sequence, extending from 667 nt to 646 nt on the opposite DNA strand (underlined in Fig. 2), showed about 86% homology to a promoter region of human rRNA gene (ACGTCGCC-TGGGCCGGCGGCGTGG, from -102 to -79 nt in ref. 8, Fig. 3, where +1 is the transcription initiation point).

```
GTGACACGGAGGGAGGTCATCGCCGACTTCACCGAGCCTGGGGCAACGGGTTTCTCTCTC    60
TCCCTTCTGGAGGCCCCTCCCTCTCTCCCTCGTTGCCTAGGGAACCTCCGCCCTGCGGCC   120
CTATTGTTCTTTGATCGGCGCTTTACTTTTCTTTGTGTTTTGGCGCCTAGACTCTTCTAC   180
TTGGGCTTTGGGAGGGTCAGTTTAATTTTCAGTTGCCCCCGCTCCCCCATACCAGTCCTT   240
CACCTAATTTTAGTGAGTCGGTTAAGGTGGTTCCCAACCGCCCCCCCCCCCCGCCTCCCA   300
ACACCCTGCTTGGAAACCTTCCAGAGCCACCCCGGTGCTGCCTCCGTCTTCTCTCCCCTT   360
CCCCCACCCCTTGCCGGCGATCTCATTCTTGCCAGGCTGGACATTTGGCAATCGGTGGGC   420
GTCAGGCCTCACTCGGGGGCCACCGTTTTTGAAGATGGGGGCGGCACGGTCCCACGTTCC   480
CCGGAGGCAGCTTGGGCCGATCGATAGCCCCTTACCCGCGTGGGCAAGCGGGCGGGTCTG   540
CAGTTGTGAGGCTTTTCCCCCCGCTCGTCCGCCTCAGGCCTCCTCCCCTAGGAAAGCTTG   600
ACCCTGGTGGGTCTCGGTGACTTCTATCACGATGTCCTAGTTTCTCCGCCCCTCCGGCCA   660
GGCACGCAGCCGGGACAATGCGAAGGGCGCCACGGCTCTAGTCTGGGCTTCTCAGTATTG   720
CCCAACCCTAGAACGTTTCTGAAAACTAATAACGTTTCGGTCACTTAAGATTTCCAGGGA   780
CGGCGCCTTGGCCCGTGTTTGTTGCTTGTTTTGTTTCGTTCTGTTTTGTTTTGTTCGTGT   840
TTTTCCTTTCTCGTATGTCTTTCTTTTCAGGTGAAGTAGAAATCCCCATTTTCAGGAAGA   900
CGTCTATTTTCCCCAAACACGTTAGCTCCGTTTTTTCCTTGTTGTAACTACGCTTTTGTG   960
ACTCTCTAACGTGCAGTGAGAGCCGGTTGATGTTTACTATCCTTCATCATGACATCTTAT  1020
TTTCTAGA
```

Fig. 2. The DNA sequence of the SalI-XbaI fragment of the human NTS (non-coding strand). The sequence motif homologous to the promoter region of the human rDNA is underlined.

Tjian et al. (9, 10) suggested that two promoter regions, the core promoter and the upstream control element were required for the efficient transcription initiation of human rRNA gene. In the first step of the transcription initiation both promoter elements are recognized by a protein factor (UBF1) and this protein factor binds to a DNA region between -75 and -114 nucleotides (11, 12). As it was mentioned above a part of the SX region showed high homology to a part of the binding sequence from -79 to -102. This suggests that the homologous NTS region may also bind the same transcription factor (UBF1) and may act as a spacer promoter.

The spacer promoters were first found in the NTS of Xenopus laevis rDNA. These NTS regions showed homology to the promoter sequence of Xenopus rDNA and seemed to play a very important role in the regulation of Xenopus rDNA transcription (13). Recently, spacer promoters were also detected in the NTS of rat and mouse rRNA genes (14, 15, 16). Nobody succeeded, however, in finding a spacer promoter in the NTS of human rDNA. Therefore the results described in this paper may be important in the elucidation of structure and understanding the function of the NTS of human rDNA.

To sum up, nucleotide sequence of a region of the non-transcribed spacer (NTS) of human rRNA gene was determined. The sequenced region, designated as SX, is close to the second XbaI element of the NTS, already described earlier (1). Part of this region shows sequence homology to a promoter region of the human rRNA gene. Authors suggested that this sequence may bind transcription initiation factors and act as a promoter sequence in the non-transcribed spacer of the human ribosomal RNA gene.

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Short paper

INHIBITION OF CYTOCHROME C OXIDASE BY GLUTATHIONE IN VITRO

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The in vitro interaction of low molecular weight thiols with heme containing enzymes has been the subject of several studies. Glutathione (GSH) binds to protoporphyrin IX iron (III) (Silver and Lukas, 1985). Thiols inhibit the ferric hemoprotein catalase (E.C. 1.11.1.6.; Takeda et al., 1980; Jouve et al., 1984; Jouve et al., 1986) and horseradish peroxidase (E.C. 1.11.1.7.; Jain, 1982). The latter enzyme can catalyze the oxidation of thiols, both in oxygen- (Olsen and Davis, 1976; Harman et al., 1984) and in peroxide-consuming (Harman et al., 1986; Medeiros et al., 1987) reactions. Thiyl radical formation was observed during the oxidation of cysteine (Harman et al., 1984) and GSH (Harman et al., 1986). However, several authors have found that GSH is a very poor substrate for heme-containing peroxidases, and they applied various mediators to promote the oxidation (Nakamura et al., 1986; Svensson, 1988). The peroxidase-generated free radical metabolites of various drugs and toxic chemicals can also oxidize GSH to its thiyl radical (Ross et al., 1985; Subrahmanyam, 1987).

Cytochrome C oxidase (E.C. 1.9.3.1.) contains four metal atoms per unit, i.e. two iron atoms in heme and two copper atoms. Sulphide ion is a known slow-binding inhibitor of cytochrome C oxidase (Nicholls, 1975), it forms a low-spin heme sulphide compound with the oxidized enzyme (Wilson et al., 1976).

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The present study was carried out to investigate the in vitro interaction of glutathione with cytochrome C oxidase.

Bovine hearth cytochrome C oxidase and cytochrome C (type III from horse heart) were obtained from Sigma (St. Louis, USA) and were used as received. Cytochrome C was reduced by sodium dithionite and separated from excess reductant by gel filtration on Sephadex G-25 (Pharmacia, Uppsala, Sweden) in 0.1 M Na-phosphate buffer, pH 7.4. The ferrocytochrome C concentration was calculated using ε_{red} = 27.7 $mM^{-1}cm^{-1}$ at 550 nm (Margoliash, 1954). Cytochrome C oxidase (21 nM) was suspended in 1.2 ml of 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1 mM EDTA-Na_2 and 0.5% Tween 80 (buffer A) and it was preincubated with GSH (0-1 mM). After various time periods aliquots were taken and the enzymatic activity was determined. The concentration of cytochrome C oxidase was determined according to Van Gelder (1966). GSH was removed from the preincubation mixture by molecular sieving (Centrisart I, SM 132 49, Sartorius, Göttingen, F.R.G.).

The cytochrome C oxidase activity was measured spectrophotometrically at 550 nm. The assay mixture consisted of 30 µM ferrocytochrome C, aliquot from the preincubation mixture and buffer A in 2.5 ml final volume. The initial velocities were determined at 25°C, by using the absorption coefficient $\Delta(\varepsilon_{red}-\varepsilon_{ox})$ = 18.5 $mM^{-1}cm^{-1}$ for cytochrome C (Margoliash, 1954).

The chemical reduction of air-oxidized cytochrome C (30 µM) by GSH (0.004-0.24 mM; or by 2-mercaptoethanol) was followed spectrophotometrically at 550 nm in 2.5 ml buffer A.

For each experiment three independent parallel determinations were carried out.

Thiols interfere in the cytochrome C oxidase assay since they reduce ferricytochrome C (Paul, 1955). However, it was found in the present experiments that up to 0.05 mM concentration glutathione or 2-mercaptoethanol can not influence significantly the absorbance changes at 550 nm during the assay period (approx. 5 min), because the rate of the chemical reduction of cytochrome C by these thiols up to this concentration is negligibly low.

The presence of thiols up to 0.05 mM concentration in the cytochrome C oxidase assay mixture had no effect on the enzyme activity. Higher thiol concentrations can be applied during preincubations. The preincubation of the enzyme with GSH resulted in a slow, time- and concentration dependent inhibition of the enzymatic activity (Fig. 1). The reversibility of the

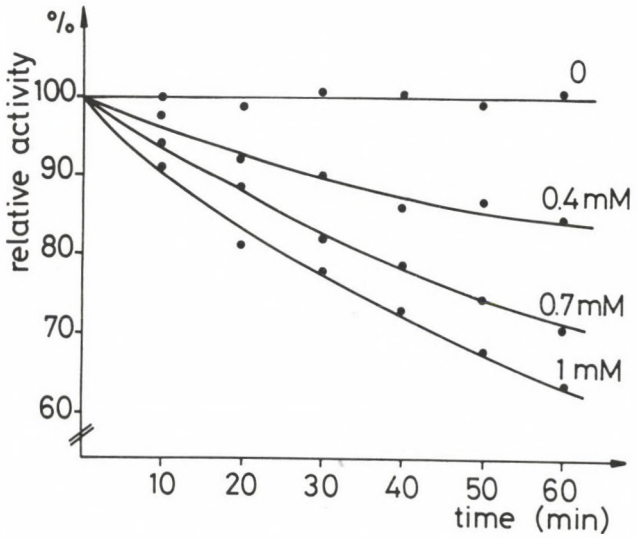

Fig. 1. The inhibition of cytochrome C oxidase with glutathione at 25°C. The enzyme (21 nM) was incubated with glutathione at various concentrations (0, 0.4, 0.7 and 1 mM) in 1.2 ml of 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1 mM EDTA-Na_2 and 0.5% Tween 80. After various time periods aliquots (100 µl) were taken and the activity was assayed. Results are given as percentage of the control activity (48 sec^{-1}, expressed in turnover number).

inhibition was checked after 3 hours of preincubation with GSH (1 mM). The residual activity was 44% of that of the control after this period. After the removal of GSH from the preincubation mixture the activity was raised to 79%. Similar phenomenon, a partial recovery of the activity after thiol inhibition was observed with catalase (Takeda et al., 1980). The preincubation of cytochrome C oxidase with 0.4 mM 2-mercaptoethanol decreased the activity by 29% during 1 hour.

These results show that the major endogenous non-protein thiol compound glutathione binds slowly but tightly to the enzyme in vitro.

In the presence of GSH (10 mM) the light absorption of oxidized (resting) cytochrome C oxidase (171 nM) slightly decreased in the Soret region (by 6% at 416 nm in 30 min) and the Soret peak was shifted from 416 to 418 nm during this period. As the spectrum of oxidized cytochrome C oxidase is unstable (Fabian and Malmström, 1989), the enzyme was incubated for 2 hours before the addition of GSH. Glutathione altered to a lesser extent the spectrum than sulphide did (Nicholls, 1975). Further studies are necessary to prove that glutathione binds to a heme iron of the enzyme. Thiols can modulate various enzymatic activities also by thiol-disulfide interchange reactions (Brigelius, 1985). The binding of an internal sulfur compound to a heme group of cytochrome C oxidase in suspensions of submitochondrial particles was suggested on the basis of EPR studies (Wilson et al., 1976). Nevertheless, the importance of the interaction between thiols and the heme iron of various enzymes in vivo and the role of this interaction in the enzyme regulation remains still to be elucidated.

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POLYADENYLATED RNAs AS ERROR SOURCES IN RIBOSOMAL RNA TURNOVER ANALYSES

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SUMMARY

An approach to ribosomal RNA turnover studies in which cytoplasmic RNA was extracted and subsequently fractionated to isolate ribosomal RNA is reported. The presumption that the pool of 28S and 18S RNAs represented ribosomal RNA, exclusively, proved false and led to erroneous results of ribosomal RNA turnover. Polyadenylated RNAs exhibited a heterogeneous size distribution and, although constituting only 3% (w/w) of the cytoplasmic RNA extract, accounted for fully 10% of radioactivity of the presumptive ribosomal RNA pool. Profiles from the radioactivity data suggested that the discrepant results were due to these polyadenylated RNAs. An additional analytical procedure, an oligo (dT) cellulose column chromatography of the RNA extract prior to the sucrose density gradient fractionation step, performed as described in this paper, proved an effective remedy for this error.

INTRODUCTION

Nucleic acid turnover analyses are powerful tools for charting drug-induced regulation of transcriptional and translational events (1-3). Cytoplasmic RNA turnover studies often rely on assays of uptake of radiolabelled RNA precursors. In our experience, it was noted, in agreement with others, that maximal labelling in most rat tissues is achieved within 48h of an intraperitoneal injection of (5-^{3}H) orotic acid into the animal (4,5). Subsequently, a progressive fall occurred in the specific radioactivity of the RNA, followed a first order kinetics, and was the basis for calculating the RNA turnover rate (3).

In our study of ribosomal RNA turnover in rat

gastrocnemius muscle, the difficulty encountered in isolating pure ribosomes in sufficient yield from this tissue prompted an approach in which intact cytoplasmic RNA was first extracted and, thereafter, subjected to sucrose density gradient centrifugation to isolate ribosomal RNA. In those series of experiments in which radiolabelled 28S and 18S RNAs from the sucrose gradient were pooled as ribosomal RNA the anticipated fall in specific radioactivity of the ribosomal RNA which should have followed maximal labelling of the nucleic acid did not occur. This observation is inconsistent with the general concept of uptake of labelled precursors into RNA, breakdown of the labelled nucleic acid, and a consequent fall in the specific radioactivity of this nucleic acid. Investigations to explain and remedy this apparent discrepancy are reported and discussed here.

MATERIALS AND METHODS

RNA extraction and quantitation: RNA was isolated by phenol extraction from the gastrocnemius muscle of male Wistar rats at the specified time intervals after a single intraperitoneal injection of 20μCi (5-^{3}H) orotic acid (Radiochemical Centre, Amersham, U.K.) into the animal, and subjected to sucrose gradient fractionation, all according to methods previously described elsewhere (6). The nucleic acid was quantitated using the orcinol assay method (7) or, in the case of very dilute solutions, by ultraviolet absorbance measurements at 260nm against the standard molar extinction coefficient at this wavelength (8). Each assay was performed in duplicate and recorded results were mean values.

Isolation of Polyadenylated RNA: The RNA from the phenol extraction step was dissolved in 5ml 0.01M Tris-HCl buffer, pH 7.5 at 20°C containing 0.001M EDTA to remove any traces of Mg^{++} which could cause RNA aggregation. The solution was then made 0.5M with respect to KCl and applied to a small glass column containing 0.4g oligo (dT) cellulose (Sigma Chemical Co. Poole, U.K.), cellulose with a mixture of polythymidic acid chains up to 18 nucleotides long and covalently attached via the 5' phosphoryl end. This modified cellulose was loaded as a suspension in the application buffer (0.01M Tris-HCl, pH 7.5 at 20°C and containing 0.5M KCl) and packed under gravity in a 2ml - capacity column plugged with cotton wool. The column was washed with 20ml of the application buffer and both the effluent and washings were collected as polyadenylate-free RNA. Material retained on the column was

eluted with 20ml 0.01M Tris-HCl buffer, pH 7.5 at '20°C, and collected as polyadenylated RNA. Where radioactivity counts were required, the polyadenylated RNA was precipitated from its solution by first making it 0.2M with respect to NaCl, adding 2 vol cold ethanol, and standing the preparation at -15°C overnight to allow precipitation of the RNA. This precipitation was aided by addition of 1.0mg yeast RNA (bearing no radioactive label) as a carrier.

Radioactivity measurements of nucleotides: All radioactivity measurements were performed by liquid scintilltion using an NE250 scintillator (Nuclear Enterprises, U.K. Ltd.) on an Intertechnique spectrometer as described by Goodlad and Onyezili (9). To estimate the radioactivity in gastrocnemius muscle nucleotide pool, the tissue was homogenised in 5 vol 0.3M KCl at 0°C and the nucleotides were extracted from this homogenate with an equal volume of 0.4M perchloric acid at 4°C (4). The acid-soluble pool was neutralized by titration with 0.6M KOH and the perchlorate-free supernatant, obtained by centrifugation at 1000 x g for 15min at 4°C, was passed through a column (9cm 5cm) of Abrelite resin anion exchanger (CG 400, Type II, 200 mesh) which retained the nucleotides via their negatively charged phosphate groups. Subsequently, the nucleotides were eluted with 20ml of 1.0M HCl and the eluate was subjected to repeated vacuum distillation at 50°C to remove the acid. The residue, dissolved in 2.0ml of 0.1M KOH, was counted for radioactivity. All radioactivity counts were performed in duplicates and mean values reported.

RESULTS AND DISCUSSION

Given the limited facilities available in some laboratories, the technique of RNA - DNA hybridization for the assay of ribosomal RNA, although very wide spread may be handy and less sophisticated methods are resorted to. Also, although it would seem self-evident that if the turnover of ribosomal RNA is to be examined the material should be purified first, shortfalls in purification may be encountered, predicated in the method adopted for this purpose. Where pure ribosomes are isolated after a dose of radiolabelled orotic acid and used for RNA turnover studies, the calculated rates are those of ribosomal RNA (4). Where, as was experienced with rat gastrocnemius muscle, pure ribosomes are difficult to extract in suitable yield, an alterantive approach is to extract undegraded cytoplasmic RNA and, subsequently, to isolate the ribosomal fraction on a sucrose density gradient by

centrifugation. This approach assumes that the pool of 28S and 18S RNA peaks on the gradient fractionation profile represents ribosomal RNA and that other RNA types (including those with poly A tails) have different sedimentation coefficients. For rat gastrocnemius muscle cytoplasmic RNA, the results of investigations reported here suggest that this assumption is false. Results of sucrose gradient analyses presented in Fig.1 indicated, as expected, that most of the cytoplasmic RNA extracted from the tissue was ribosomal (i.e. of the 28S and 18S type). RNAs with poly A tails constituted, as evident from results in Table 1, only 3% (w/w) of the cytoplasmic RNA

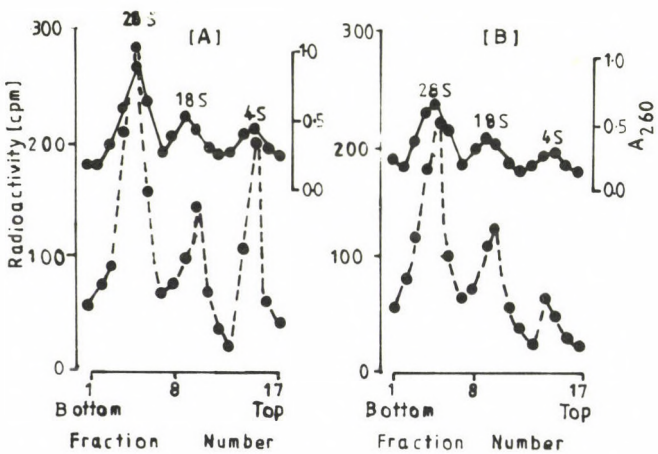

Fig. 1. Radioactivity profiles of 28S, 18S, AND 4S RNAs from rat gastrocnemius muscle RNAs were isolated from the tissue 48h after a single intraperitoneal injection of 20μCi (5-^{3}H) orotic acid into the animal and the cytoplasmic extract was fractionated by sucrose density gradient centrifugation at 5°C for 7h at 60,000xg. Broken lines indicate radioactivity levels and unbroken lines represent A_{260}. In graph [A], no oligo (dT) cellulose chromtography was performed on the sample. In graph [B], the cytoplasmic RNA extract was chromatographed on an oligo (dT) cellulose column, as described inthe text, to remove polyadenylated RNAs before subjecting the sample to sucrose density gradient centrifugation.

extract. However, a comparison (Table 1 and Fig.1) of specific radioactivities showed that the polyadenylated RNAs accounted for fully 10% of the radioactivity in the presumptive ribosomal RNA pool (i.e. the pool of 28S and 18S fractions).

Table 1. Distribution of radioactivity between polyadenylated and other cytoplasmic RNAs in rat gastrocnemius muslce

Each animal received a single intraperitoneal injection of 20μCi (5-^{3}H) orotic acid and was sacrificed 48h later. Polyadenylated RNA was isolated from other cytoplasmic RNA in the extract by chromatography on an oligo (dT) cellulose column column as described in the text.

	RNA content (mg/g tissue)	Specific Radioactivity (cpm/μg RNA)
Polyadenylated RNA	0.04	0.74
Other RNAs	1.38	0.23

Thus, the contribution from polyadenylated RNAs to the radioactivity data from the presumptive ribosomal RNA pool exceeded, quite significantly, stoichiometric expectations.

Data presented in Table 2 show radioactivity levels in the presumptive ribosomal RNA pool, isolated from the tissue and assayed prior to the exclusion of polyadenylated RNAs. The progressive fall in specific radioactivity levels of the 'ribosomal' RNA, expected after the initial 48h did not occur. Rather, the observed pattern paralleled that obtained with the polyadenylated RNA fraction in which specific radioactivity levels also failed to fall after 48h (Table 2). This picture would suggest that the abnormality in the 'ribosomal' RNA radioactivity profile was due to the contribution from the

Table 2. Radioactivities of 'ribosomal' RNA, polyadenylated RNA, and the nucleotide pool in rat gastrocnemius muscle

A single dose of 20μCi (5-^{3}H) orotic acid was injected intraperitoneally into each animal which was sacrificed on the days indicated. Extraction of, and measurements of radioactivities in, the various RNAs and the nucleotide pool were performed as described in the text. Each experiment was performed in duplicate and results are mean values from two experiments.

Days after injection of [^{3}H] orotic acid	1	2	4	6	8	10
Radioactivity of 'ribosomal' RNA (cpm/μg RNA)	-	0.88	0.99	1.12	1.25	1.29
Radioactivity of polyadenylated RNA (cpm/μg RNA)	-	0.93	1.08	1.14	1.23	1.24
Radioactivity of nucleotide pool (cpm/g muscle x 10^{-3})	16.1	2.2	1.2	1.2	1.0	0.9

polyadenylated RNAs. In interpreting these results, it was considered that radiolabelled nucleotide products of catabolized radiolabelled RNAs in the RNA precursor pool were re-utilized to sustain the radioactivity levels observed over the experimental period. However, as results of measurements of radioactivity levels in the nucleotide pool indicate in Table 2, after the fall (which occurred within 48h) in the radioactivity level of this pool, the relatively very low radioactivity of the pool remained fairly constant over the experimental period. In no instance was an increased radioactivity recorded after the 48h following the administration of the radioactive label. This observation would support the view that any such reutilization of radiolabelled RNA precursors was minimal and was not the explanation for the absence of the expected fall in radioactivity levels of ribosomal RNA following maximal radiolabelling, which should have been achieved within 48h.

When polyadenylated RNAs had been removed, by the introduction of the oligo (dT) cellulose column chromatography step as described under 'Materials and Methods', and their contribution to radioactivity counts of the 28S and 18S RNA pool thus nullified, the specific radioactivity of ribosomal RNA fell progressively and followed first order kinetics (Fig. 2) over the experimental period. Thus, by including this additional chromatography step, an acceptable basis was afforded for computing ribosomal RNA turnover rates in the rat gastrocnemius muscle by the technique described.

The polyadenylated tails found in most eukaryotic cells are not of ribosomal origin, are not required for translocation to the cytosol or for protein synthesis, and have longer half-lives than ribosomal RNA (10-12). In the rat gastocnemius muscle, as results presented in Fig. 1 showed, polyadenylated RNAs exhibited a heterogeneous size distribution and appeared in sucrose density gradient fractions erroneously attributed to ribosomal RNA exclusively. We are led to conclude that the presence of polyadenylated RNAs was responsible for the absence of a fall in the specific radioactivity of 'ribosomal'

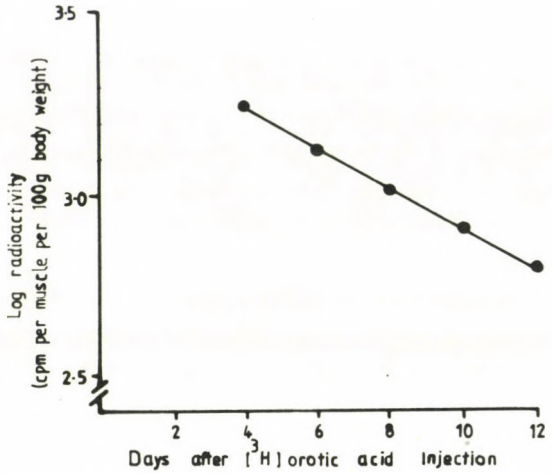

Fig. 2. Loss of radioactivity in rat gastrocnemius muscle ribosomal RNA
The animals each received a single dose of 20μCi (5-^{3}H) orotic acid intraperitoneally and were sacrificed on the days indicated. Undergraded cytoplasmic RNA was isolated from the tissue, freed of polyadenylated RNAs as described in the text and, thereafter, subjected to sucrose density gradient fractionation to obtain the ribosomal RNA pool which ws counted for radioactivity. Each point on the graph represents the mean value from 4 experiments and the slope was plotted by method of "least squares".

RNA in this tissue 48h after injecting labelled orotic acid into the animal. The inclusion, as described, of an oligo (dT) cellulose column chromatography of the RNA extract prior to the sucrose density gradient fractionation step obviated the contribution from these polyadenylated RNAs to the radioactivity data for ribosomal RNA and proved a simple and effective remedy for this error. The procedure described for isolating polyadenylated RNAs fractionates a mixture of polynucleotides according to the ability of individual components to form base pairing complexes with the complimentary oligo deoxythymidylates immobilized covalently on cellulose. In a medium of high ionic strength,

polyadenylate-containing nucleotides bind selectively and are retaineo on this modified cellulose. Upon lowering the ionic strength of the medium in the slightly alkaline conditions (pH 7.5), identical charges on adjacent molecules repel each other, enabling elution of the bound polyadenylated RNA.

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EFFECT OF PHOSPHOLIPIDS ON THYROID 5'-NUCLEOTIDASE

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SUMMARY

Phospholipids were separated from 5'-nucleotidase in thyroid plasma membranes by Sephadex G-200 gel filtration. After removal of lipids 5'-nucleotidase was still active and reassociation of the enzyme with phospholipids had a little effect on the increase of enzyme activity. Arrhenius plot of the 5'-nucleotidase activity in native thyroid plasma membranes clearly exhibited a break at 28°C. Biphasic nature of Arrhenius plot showed that the enzyme activity was influenced by physical state of membrane bilayer, although phospholipids were not obligatory cofactor for this enzyme.

INTRODUCTION

It is well established that the activity of many membrane bound enzymes is influenced by phospholipids. Phospholipids may act as modulators of enzymatic reactions in addition to their role as obligatory cofactors for some membrane enzymes (1). 5'-Nucleotidase (E.C. 3.1.3.3., 5'-ribonucleotide phosphohydrolase) is a glycoprotein ectoenzyme which is tightly bound to the plasma membranes of mammalian cells (2, 3, 4). This enzyme can be solubilized by treatment of membranes with variety of ionic and non-ionic detergent (5, 6). The ability to solubilize 5'-nucleotidase with the phosphatidylinositol-specific phospholipase C suggests that phosphatidylinositol is involved in the attachment of 5'-nucleotidase to the membrane (7, 8). An examination of a possible association of lipids and 5'-nucleotidase activity was prompted by the report of

Windell and Unkeless (9), who showed that the enzyme purified from liver plasma membranes and microsomal fractions was linked with sphingomyelin. Membrane bound 5'-nucleotidase has been purified to homogeneity by several investigators, however there are inconsistent informations as to the presence of phospholipids in the purified enzyme and the functional role of lipid component (2, 4, 10).

In this report the role of endogenous phospholipids in the activity of 5'-nucleotidase from pig thyroid plasma membranes was investigated. Arrhenius plot behaviour of temperature effect on 5'-nucleotidase activity was studied.

MATERIALS AND METHODS

Purification of plasma membranes. The pig thyroid glands were obtained from the slaughterhouse immediately after killing the animals. The plasma membranes were prepared according to the method described by Verrier et al. (11) with slight modifications. After removal of connective tissue and fat, the finely chopped thyroid glands were homogenized in 0.25 M sucrose, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4 (1 g slices in 2 ml buffer) in a Potter homogenizer. The homogenate obtained from 100 g glands was diluted to a final volume of 800 ml and filtered through fourfold gauze. The residual tissue fragments remaining on the gauze were rehomogenized in 200 ml buffer and filtered through fourfold gauze. Both filtrates were centrifuged at 10 000 x g for 10 min. The pellets were gently homogenized in 1 mM $MgCl_2$,10 mM Tris-HCl, pH 7.4 in a Potter homogenizer and were enriched with sucrose to obtain a final concentration of 43.3% (w/w). 15 ml of the sucrose-enriched suspension was layered over 8 ml 45% (w/w) sucrose solution and overlaid by sucrose solutions of 41% (10 ml) and 8% (5 ml). The tubes were centrifuged at 24 000 rpm/min for 2 h using SW57 rotor in VAC-601 ultracentrifuge. Plasma membranes were collected by aspiration at the interface 41%, 8%, diluted (1:6) with 10 mM Tris-HCl, pH 7.4 and centrifuged at 10 000 x g for 10 min. The pellet was suspensed in water and frozen. The plasma membranes could have been stored frozen at -20°C for several months with no loss of 5'-nucleotidase activity.

Separation of lipids from proteins. The plasma membranes solubilized in 1% sodium deoxycholate were poured on Sephadex G-200 column equilibrated with 1% sodium deoxycholate. Elution was carried out with the same solution. In all fractions 5'-nucleotidase activity, protein concentrations and phospholipid contents were measured. Fractions from column containing 5'-nucleotidase activity were pooled and deoxycholate was removed by precipitating the protein with cold acetone (-20°C). Obtained protein fraction was completely free of lipids. The application of dialysis of 5'-nucleotidase solution against

40 mM veronal buffer, pH 7.5 did not give satisfactory results because 5'-nucleotidase activity was decreasing during dialysis. Obtained lipid fractions did not show 5'-nucleotidase activity.

Enzyme assay. 5'-Nucleotidase activity was routinely determined by the method of Campbell (12) by measuring inorganic phosphate liberated from AMP. The method involves two parallel enzyme activity determinations with AMP as substrate. In one the presence of nickel specifically inhibits 5'-nucleotidase, and therefore estimates the hydrolysis of substrate by non-specific alkaline phosphatase. In the second the absence of nickel allows the estimation of total phosphatase activity. The difference in activity (in terms of inorganic phosphate liberated) gives the 5'-nucleotidase activity (12). Phosphate liberated was measured by the method of Chen et al. (13). Specific activity was expressed in μmol of released inorganic phosphate x min^{-1} x mg of $proteins^{-1}$. Protein concentration was estimated according to Lowry et al. (14) using bovine albumin as a standard.

Phopholipid assay. The determination of phospholipid in fractions from column was carried out according to the method of Yoshida et al. (15). Fractions containing phospholipids were pooled and extracted with chloroform-methanol (2 : 1; vol/vol) and separated by means of two-dimensional thin-layer chromatography (16). Lipid phosphorus was determined by method of Bartlett (17).

Incubation of 5'-nucleotidase with phospholipids. The incubation mixture contained 0.2 ml (about 0.05 mg) of protein (delipidated nucleotidase), 0.02-0.3 ml (2-30 nmoles) of phospholipids, 0.05 ml 20 mM $MnSO_4$, 0.1 ml 10 mM AMP and 40 mM veronal buffer, pH 7.5 in total volume of 1 ml. Composition of added phospholipid fractions was as follows: phosphatidyl-inositol (7.57%), sphingomyelin (12.55%), lysolecithin (3.54%), phosphatidylserine (1.08%), phosphatidic acid (3.25%), phosphatidylethanolamine (2.83%), phosphatidylcholine (69.1%). The mixture was incubated for 30 min at 37°C and then deproteinized by addition of 1 ml of 10% trichloroacetic acid. After centrifugation, the inorganic phosphate was determined by the method of Chen et al. (13). Control samples containing 5'-nucleotidase but not phospholipid were simultaneously incubated and treated in a similar manner. Phospholipids obtained from Sephadex G-200 column were extracted and evaporated to dryness. Then, after suspension in 40 mM veronal buffer, pH 7.5 and sonication for 7 min in a MSE 50W sonifer they were added into incubation mixture.

RESULTS AND DISCUSSION

Plasma membranes obtained from pig thyroid glands by discontinuous sucrose gradient centrifugation were solubilized in the presence of 1% deoxycholate sodium. The 5'-nucleotidase activity in deoxycholate increased with 20% over its initial

values (Table 1). An increase in 5'-nucleotidase activity after deoxycholate solubilization may be achieved by relieving a constraint imposed on the protein by the membrane (5). The 5'-nucleotidase is localized predominantly in the external face of the membrane (18) where are concentrated neutral and positively charged lipids: phosphatidylcholine, sphingomyelin, cholesterol (19). The preferential interactions of deoxycholate with positively charged phospholipid may be responsible for increase in the conformational flexibility and activity of the enzyme.

Table 1. 5'-Nucleotidase activity in the homogenate, membrane fraction and lipid free fraction

Fraction	Specific activity ($\mu mol \times min^{-1} \times mg^{-1}$)	Purification factor	Total activity ($\mu mol \times min^{-1}$)	Recovery (%)
Homogenate	0.0097	1.0	23.3	100
Plasma membranes	0.0545	5.6	5.4	23
Membranes solubilized with 1% sodium deoxycholate	0.0780	8.0	6.6	28
Sephadex G-200 column filtrate	0.2120	21.8	4.2	18
Acetone precipitate	0.4670	48.1	0.7	3

The solubilized membranes were applied to Sephadex G-200 column. Typical results are presented in Fig. 1. showing that column gel filtration allows complete separation of phospholipids from 5'-nucleotidase. This results agree with findings of Allan and Crumpton (20) for pig lymphocyte plasma membrane.

The 5'-nucleotidase activities in the homogenate, membrane fraction and lipid free fraction are presented in Table 1. The results show that 5'-nucleotidase is still active after

lipid removal what may indicate that phospholipids are not obligatory cofactors for this enzyme. The specific activity of 5'-nucleotidase after acetone precipitation is over 40-fold increased in comparison to homogenate. The addition of sonicated phospholipid extract to delipidated 5'-nucleotidase produced inconsiderable increase in the enzyme activity (Fig. 2). An inconsiderable increase in enzyme activity may result from the alteration of native conformation and poor protein-lipid interaction. Evans and Gurd (4) also showed that the addition of sonicated total lipid extract of brain, and purified sphingomyelin, phosphatidylcholine, phosphatidic acid to the purified enzyme from mouse liver plasma membrane produced no significant increase in enzyme activity. Specific protein-lipid interaction can influence the conformation of the protein and the resulting changes in the protein structure and temperature break point of the Arrhenius plot (3).

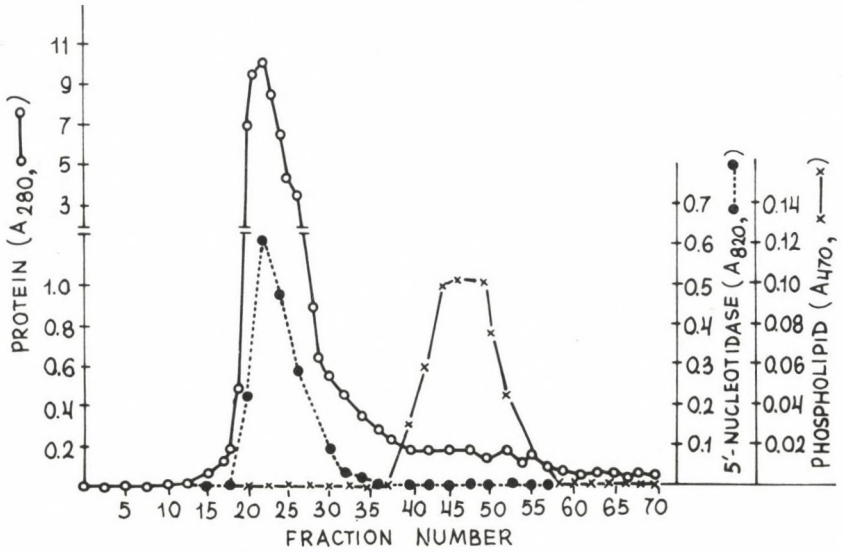

Fig. 1. Gel filtration of deoxycholate soluble fraction of plasma membrane on a Sephadex G-200 column. Plasma membrane (50 mg of protein in 6 ml of 1% deoxycholate) was added on to a Sephadex G-200 column (3x40 cm) and eluted with 1% deoxycholate. Fractions (3.4 ml) were collected and were analysed for protein (o--o), phospholipid (x--x) and 5'-nucleotidase activity (•--•).

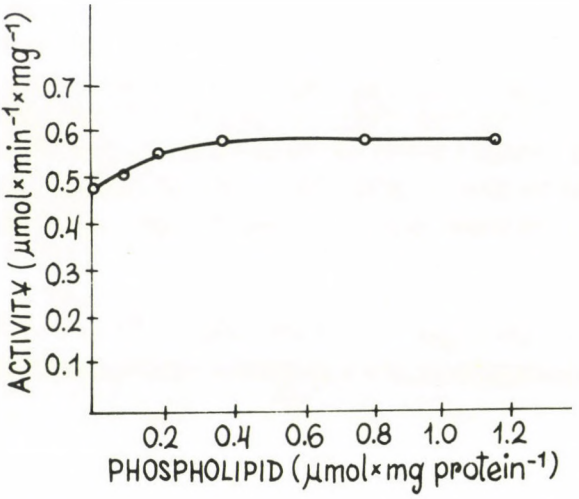

Fig. 2. Effect of phospholipids on 5'-nucleotidase activity. Delipidated 5'-nucleotidase in 40 mM veronal buffer, pH 7.5 was mixed with a suspension of phospholipids obtained from Sephadex G-200 column. The mixture was incubated for 30 min at 37°C, than enzyme activity was assayed under standard conditions (for details, see Methods).

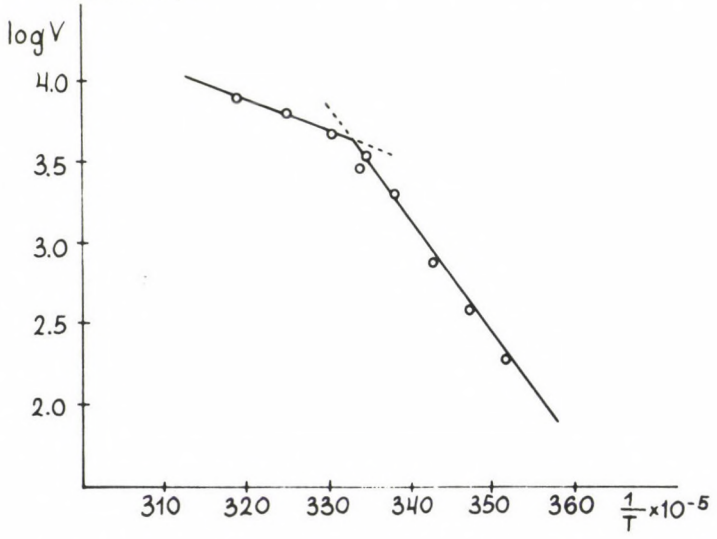

Fig. 3. Arrhenius plot of temperature effect on membrane-bound 5'-nucleotidase from pig thyroid gland.

The Arrhenius plots of the 5'-nucleotidase activity in the native membranes of pig thyroid clearly exhibit a break at 28°C (Fig. 3). The activation energy as measured from the slopes of two linear components are 126 kJ/mol below and 40 kJ/mol above the phase transition. These results show that the activity of 5'-nucleotidase is influenced by physical state of the bilayer and the enzyme operates more efficiently when the lipid is in the fluid state. Biphasic nature of the Arrhenius plot of 5'-nucleotidase has been reported by several investigators (5, 21-26). 5'-Nucleotidase from rat enterocyte membranes shows a discontinuity in the Arrhenius plot at 28-30°C, which correlates with a membrane lipids thermotropic transition detectable by differential scanning calorimetry and fluorescence polarization (26). Likewise, Arrhenius plots of the 5'-nucleotidase activity from rat liver plasma membrane exhibited a break at 28°C, although breaks were observed at higher (23) or lower temperatures (22). In contrast, the activity of 5'-nucleotidase in the lymphocyte plasma membranes shows no change in slope of Arrhenius plots (10, 27). Presumably the lipid microenvironment around a particular intrinsic enzyme and the nature of the specific lipid-protein interactions influence the break point temperature (1).

So far nothing is known about the topological organization of 5'-nucleotidase within the phospholipid bilayer. 5'-Nucleotidase may be associated with sphingomyelin in vivo since it is found in the sphingomyelin-rich bilayer of the canicule of rat liver membranes (28), and partial purification of the enzyme can yield a lipoprotein complex which contains sphingomyelin as the only phospholipid (9). Merisco et al. (23) showed that the purified 5'-nucleotidase which contained sphingomyelin as the only phospholipid exhibited neither a transition nor any other discontinuity. However, transitions were detected after reassociation of the purified enzyme with plasma membrane lipids or phosphatidylcholine but not with phosphatidylethanolamine. Authors concluded that in situ 5'-nucleotidase interacts with both sphingomyelin and phosphatidylcholine and the first influences the stability of the

enzyme and the second the energy of activation. The effect of phospholipids on stability of 5'-nucleotidase was confirmed by other workers. It was showed that the purified lymphocyte 5'-nucleotidase reconstituted into lipid bilayer demonstrates remarkable stability on storage at $4^{\circ}C$ (25). The liposome incorporated enzyme from chicken gizzard is five times more stable at $56^{\circ}C$ than the enzyme in the detergent solution indicating that the phospholipids may prevent the denaturation process (6). Thus, although 5'-nucleotidase does not require phospholipids for activity, phospholipids are highly effective at preventing denaturation of the enzyme.

Phospholipids can also influence the kinetic properties and substrate specifity of 5'-nucleotidase (2). In this study 5'-nucleotidase in the native membranes showed the highest activity towards 5'-GMP, whereas the enzyme solubilized in deoxycholate showed the highest activity towards 5'-AMP (Table 2).

Table 2. Substrate specifity of membrane 5'-nucleotidase in the presence and absence of deoxycholate (DOC)

Substrate (1 mM)	Activity (μmol Pi/mg/min)		Relative activity (%)	
	without DOC	with DOC	without DOC	with DOC
5'-AMP	0.050	0.076	100	100
5'-IMP	0.047	0.056	94	74
5'-GMP	0.061	0.070	122	92
5'-CMP	0.035	0.052	70	68
5'-UMP	0.038	0.074	76	97
5'-TMP	0.009	0.006	18	8

Each of the values is the average of the data obtained from three experiments.
The final concentration of DOC was 0.1%.

The substrate specifity of 5'-nucleotidase from various tissues is different (2, 10, 29). These differences may perhaps be due to the different lipid composition and domain structures of various membrane environments.

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THE ENZYME CAPABLE TO CLEAVE TRYPSIN ACTIVE SITE TITRANT AS SUBSTRATE IS A CARBOXYLESTERASE WITH ELECTROPHORETIC MOBILITY SIMILAR TO ALBUMIN

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SUMMARY

An enzyme isolated from Ehrlich ascites plasma and capable of cleaving trypsin active site titrant 4-nitrophenyl-p-guanidinobenzoate (Steven, F.S. and Al-Achmad, R.K. (1983) has been further investigated. The substrate hydrolysis follows Michaelis-Menten kinetics. The molecular mass of the enzyme is 50-70 kDa by gel filtration and SDS polyacrylamide gel electrophoresis. It has the mobility of albumin and coelutes with a carboxylesterase activity on a cation exchange column. $(Cbz\text{-}Arg\text{-}NH)_2$-Rhodamine, the specific noncompetitive inhibitor of guanidinobenzoatase, also inhibits the carboxylesterase activity. Therefore, the guanidinobenzoatase activity of Ehrlich ascites plasma is a carboxylesterase (EC 3.1.1.1.) which likely originates from blood.

INTRODUCTION

Steven and Al-Achmad (1983) found an enzyme called guanidinobenzoatase in the ascitic fluid surrounding Ehrlich ascites cells. The enzyme cleaved 4-nitrophenyl-p-guanidinobenzoate, on active-site titrant for trypsin, as substrate. It was

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Abbreviations: BzArgOEt, Benzoyl-L-arginine ethylester; Cbz, benzyloxycarbonyl; $(Cbz\text{-}Arg\text{-}NH)_2$-Rhodamine, bis-(N-Cbz-L-argininamido)-Rhodamine; DFP, diisopropyl fluorophosphate; EDTA, ethylene diamine tetraacetic acid; PMSF, phenylmethanesulphonyl fluoride; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate

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thought that this enzyme is produced by, or associated with tumor cells (Steven and Al-Achmad, 1983). This enzyme was also found on the surface of human malignant cells (Steven and Al-Achmad, 1985; Steven et al., 1988) and since it was able to cleave fibronectin, its involvement in cell migration was suggested (3, 4). $(Cbz\text{-}Arg\text{-}NH)_2$-Rhodamine, designed as a fluorogenic substrate for trypsin-like enzymes (Leytus et al., 1983) is a specific noncompetitive inhibitor of guanidinobenzoatase activity (Steven et al., 1988; Steven et al., 1986). A very similar activity preferring guanidinophenyl residue at the cleavage site of amide substrates was also found in Ehrlich ascites plasma (Tsunematsu et al., 1985). This enzyme is also present in human gastric and colon cancer cells (Tsunematsu et al., 1985).

The activity of cell surface proteinases seem to have a crutial role in degradation of extracellular matrix in tumor invasion (Tryggvason et al., 1987) and guanidinobenzoatase has been considered as a new candidate for such processes. Since this enzyme is poorly characterized, further investigation has been initiated.

MATERIALS AND METHODS

The following materials were obtained from the indicated sources: Ammonium persulfate from Merck (Darmstadt, FRG), Coomassie Brillant Blue R-250, SDS from Bio-Rad (Richmond, Ca., USA), 9-aminoacridine, benzamidine, DFP, iodoacetamide, SBTI, SDS electrophoresis standard, PMSF from SIGMA (St. Louis, Mo., USA). D-Val-Leu-Lys-pNA and D-Phe-Pro-Arg-pNA from KABI (Mölndal, Sweden), cellogel strips from Chemetron (Milano, Italy). $(Cbz\text{-}Arg\text{-}NH)_2$-Rhodamine and human fibronectin were generous gifts of Dr. F.S. Steven, Dept. of Biochemistry, University of Manchester, U.K. and Dr. V. Koteliansky, Cardiology Res. Center, Academy of Med. Sciences, Moscow, USSR, respectively.

The Ehrlich ascites tumor-bearing mice were kindly supplied by Dr. P. Kertai, from the Department of Hygiene and Epidemiology. Tumor cells and ascites plasma were prepared according to Tsunematsu et al. (1985). The membrane of Ehrlich ascites cells was prepared according to Spitzer et al. (1983).

The guanidinobenzoatase assay was carried out in 1 ml 0.1 M Na-barbiturate buffer, pH 8.3, at 30°C and the liberated p-nitrophenol was calculated using $\varepsilon_{410} = 16\ 595$ (Chase and Show, 1967).

Cellulose acetate membrane electrophoresis was performed in 0.05 M Na-barbiturate buffer, pH 8.3. The membrane was stained for guanidinobenzoatase activity as follows. Electrode buffer containing 0.7 mM substrate was applied to the cellulose-acetate membrane. After 20 min of incubation at 37°C in a moist chamber, a yellow line appeared and its place was marked with pencil, the membrane was then stained with Amido Black 10 B in methanol:acetic acid:water (5:4:1).

Gel filtration was carried out on Superose-6 HR 10/30 column using FPLC equipment (Pharmacia, Uppsala, Sweden). 200 µl dialyzed sample was applied and elution was performed with 0.1 M phosphate buffer, 0.15 M NaCl, pH 7.0. The flow rate was 0.4 ml/min and 1 ml fractions were collected. The column was calibrated with Pharmacia high and low molecular weight gel-filtration standards.

Cation exchange chromatography was performed with FPLC on a MONO Q HR 5/5 column (Pharmacia). The column was equilibrated with 0.05 M Tris-HCl buffer, pH 8.5 (buffer A) and 100 µl dialyzed ascites plasma was applied. Buffer B contained 1.0 M sodium chloride in buffer A. The flow rate was 0.5 ml/min and 1 ml fractions were collected.

Carboxylesterase activity was measured using α-naphtyl-acetate and fast blue RR (Robbi and Beaufay, 1983).

Protein was determined according to Bradford (1976). SDS-PAGE was prepared according to Laemmli (1970).

The fraction from the MONO Q column containing the highest guanidinobenzoatase activity was tested for fibronectin cleaving ability as follows: 8 µg protein of the peak fraction was incubated with 33 µg human plasma fibronectin in 200 µl 50 mM Tris-HCl, 110 mM NaCl, pH 7.4, at room temperature and aliquots were removed every 24 hours for 3 days and tested using SDS-PAGE.

RESULTS AND DISCUSSION

In accordance with the results of Steven and Al-Achmad (1983), the ascites plasma cleaved p-nitrophenyl-guanidino-benzoate as a true substrate. The reaction followed Michaelis-Menten kinetics (Fig. 1a) with K_M = 0.08 mM and V = 2.5 µM s^{-1} (0.3 nmol mg^{-1} s^{-1} maximum specific activity). The initial velocities were proportional to the amount of the ascites plasma (Fig. 1b).

The effect of various proteinase inhibitors on guanidino-benzoatase activity is summarized on Table 1. The activity was inhibited by DFP and PMSF supporting the presence of a serine residue in the active site (Steven and Al-Achmad, 1983). However, though arginine selectivity of the enzyme was reported (Steven and Al-Achmad, 1983), the plasmin substrate D-Val-Leu-

Lys-pNA was also a good inhibitor of this activity, in a competitive manner (data not shown).

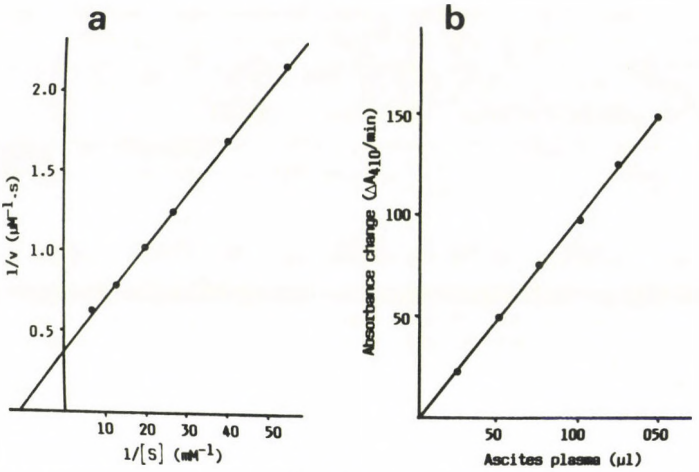

Fig. 1. Lineweaver-Burk plot (a) and concentration dependence (b) of guanidinobenzoatase activity of Ehrlich ascites plasma. Activity was measured in 0.1 M Na-barbiturate buffer, pH 8.3 at 30°C. The substrate concentration was 0.02-0.15 mM (a) as well as 0.05 mM (b).

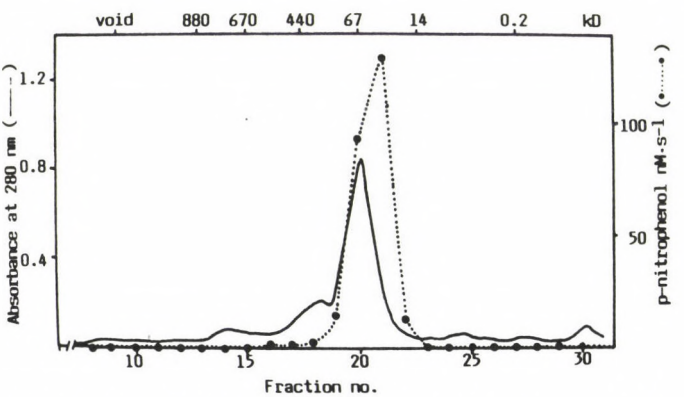

Fig. 2. Gel filtration of Ehrlich ascites plasma on Superose 6B HR 10/30 column. The column was calibrated with the following materials (kDa in parentheses): ferritin dimer (880); thyroglobulin (670); ferritin (440); bovine serum albumin (67); ribonuclease A (14) and cytidine (0.2). Fractions were tested for guanidinobenzoatase activity as described in Materials and Methods.

Table 1. Effect of various preteinase inhibitors on guanidinobenzoatase activity of Ehrlich ascites plasma

Inhibitors were preincubated with 50 µl ascites plasma in 0.1 M Na-barbiturate buffer, pH 8.3 for 60 min at room temperature then the remaining activity was measured at 30°C by the addition of 0.1 mM p-nitrophenyl-guanidinobenzoate

Inhibitor	Concentration	Remaining activity (%)
DFP	1.0 mM	5
PMSF	1.0 mM	18
Iodoacetamide	1.0 mM	107
$HgCL_2$	2.0 mM	70
EDTA	10.0 mM	95
Benzamidine-HCl	1.0 mM	97
SBTI	5.0 µM	96
BzArgOEt	0.1 mM	90
D-Val-Leu-Lys-pNA	0.3 mM	60
D-Phe-Pro-Arg-pNA	0.5 mM	50
9-Aminoacridine	10.0 µM	76
$(CBz\text{-}Arg\text{-}NH)_2$-Rhodamine	1.0 µM	30

The molecular mass of guanidinobenzoatase was 50 kDa by gel filtration (Fig. 2). However, after preparation by agmatine-Sepharose affinity chromatography, the enzyme was identified as two protein bands with 68 and 70 kDa by SDS-polyacrylamide gel electrophoresis (Steven et al., 1988b).

Ehrlich ascites plasma was subjected to cation exchange chromatography on MONO Q and the fractions were tested both for guanidinobenzoatase and carboxylesterase activity (Fig. 3). The highest esterase activities were found in the same fraction, at the major protein peak (albumin) of the ion exchange chromatography(Fig. 3). This fraction was subjected to SDS-polyacrylamide gel electrophoresis (Fig. 3, inset): two protein bands were found both in reducing and non-reducing conditions, at 67 and 50 kDa as well as 60 and 50 kDa, respectively. Though guanidinobenzoatase was identified also as two proteins after ag-

matine-Sepharose affinity chromatography (3), it is still uncertain whether both proteins have activity or one of them is only a contaminant (likely albumin). Our peak fraction was unable to cleave fibronectin at the conditions described in Materials and Methods (data not shown).

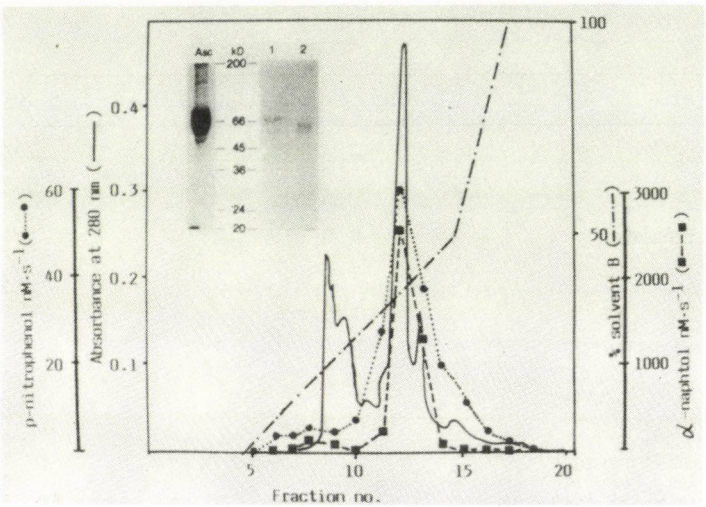

Fig. 3. Ion exchange chromatography of Ehrlich ascites plasma on MONO Q HR 5/5 column. Fractions were tested for guanidinobenzoatase activity and carboxylesterase activity as described in Materials and Methods. Inset shows the SDS-polyacrylamide gel electrophoresis of the peak fraction in reducing (1) and non-reducing (2) conditions. Ehrlich ascites plasma (Asc) was also run in reducing conditions.

The guanidinobenzoatase and carboxylesterase activity of the MONO Q fraction with highest activities were compared (Table 2). Both activities were inhibited by DFP. Furthermore, the carboxylesterase activity was also inhibited by (Cbz-Arg-NH)$_2$-Rhodamine, a specific noncompetitive inhibitor of guanidinobenzoatase (Steven et al., 1986). Taken into account the similar aromatic nature of guanidinophenyl and α-naphtyl residues, the substrate binding site of the esterase may easily accept them.

Guanidinobenzoatase had albumin mobility during cellulose acetate membrane electrophoresis (Fig. 4), similarly to that of albumin esterase of mouse plasma (Talal et al., 1963). Mouse

Table 2. Comparison of the guanidinobenzoatase and carboxylesterase activity of the fraction from MONO Q column with the highest esterase activity (Fig. 4, fraction No. 12)

	REMAINING ACTIVITY %	
	Guanidino-benzoatase activity	Carboxyl-esterase activity
1 mM DFP	7	0
1 μM $(Cbz\text{-}Arg\text{-}NH)_2$-Rhodamine	17	15

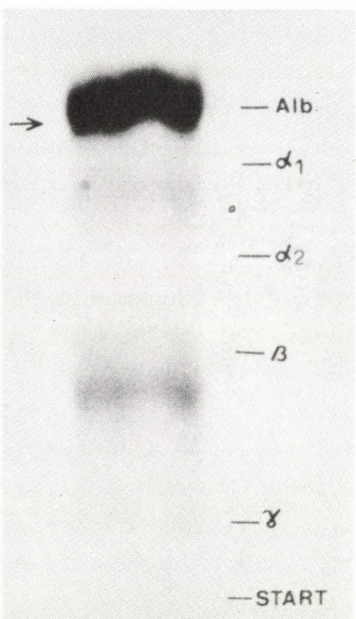

Fig. 4. Cellulose acetate membrane electrophoresis of Ehrlich ascites plasma. The mobility of the bands was identified by running human plasma simultaneously. The membrane was developed for guanidinobenzoatase activity as described in Materials and Methods. The place of guanidinobenzoatase activity was marked by pencil (it is indicated by an arrow). Then the membrane was stained with Amido Black 10B.

plasma contains guanidinobenzoatase activity in even higher activity than ascites plasma (Steven and Al-Achmad, 1983). Since there was no measurable guanidinobenzoatase activity in cell lysate as well as in membrane preparation of Ehrlich ascites cells (data not shown), the ascites cell origin of guanidinobenzoatase is doubtful.

Considering the similar molecular weight, electrophoretic mobility, the common substrate (α-naphtylacetate) and inhibition by $(Cbz\text{-}Arg\text{-}NH)_2$-Rhodamine, it is very likely that the guanidinobenzoatase activity of Ehrlich ascites plasma is due to the activity of albumin esterase found in mouse plasma which was also inhibited by DFP (Talal et al., 1963). Albumin esterase was originally thought to be an example of the microheterogenity of albumin (Talal et al., 1963) since albumin itself has an esterase activity inhibited by DFP due to the diisopropylphosphorylation of a reactive tyrosine side chain (Means and Wu, 1987). However, Popp et al. (1966) purified the albumin esterase and found its amino acid composition different from that of albumin.

It is still remains obscure whether this enzyme is identical to the GPASE reported by Tsunematsu et al. (1985). Neither amidolytic nor fibronectin cleaving ability of guanidinobenzoatase was verified in our studies. However, presence of guanidinobenzoatase at the surface on human tumor cells and the possibility of specific activity staining (Steven and Al-Achmad, 1985; Steven et al., 1986; Steven et al., 1988a; Steven et al., 1988b) warrants further research to clarify its possible involvement in cell migration or its usefulness for diagnostic purposes.

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SIMILARITIES AND DISSIMILARITIES BETWEEN PLANT UsnRNAs AND THEIR EQUIVALENTS IN OTHER EUKARYOTES WITH RESPECT TO STRUCTURE, COMPLEXING WITH PROTEINS, GENE ORGANIZATION AND FUNCTION

A REVIEW

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Introduction

The last decade has witnessed an exceptionally dynamic progress in the study of uridylate-rich small nuclear RNAs (UsnRNAs; *cf. 26, 68*), their genes (*cf. 16, 66*) and the nucleoprotein particles (UsnRNPs; *cf. 52, 96*) they are components of. Certainly, the main reason of this increased research activity lies in the fact that UsnRNAs, in the form of nucleoprotein particles, the UsnRNPs, were found to be indispensable *trans*-acting factors in the splicing (U1, U2, U4 to U6 RNAs) of nuclear pre-mRNA (*cf. 26, 53, 75*) and in the processing (U3 RNA) of nucleolar pre-rRNA (*cf. 33*).

In general terms, splicing of pre-mRNA consists of the removal of its introns (IVS, intervening sequence) as lariat structures and the ligation of its exons to give rise to mature, functional mRNA. In this process (i) consensus sequences at both the 5' and 3' splice sites (5' ss and 3' ss) of pre-mRNA, (ii) a well (yeast) or loosely (vertebrates) defined consensus sequence, the branch site (BS) around the branch point (BP, an A residue) of the intron, (iii) UsnRNPs, and (iv) a number of protein factors (see Caption to Fig. 1) are involved. A simplified sketch of pre-mRNA splicing in multicomponent RNP complexes termed spliceosomes and known to assemble *in vitro* in both HeLa (*22*) and yeast (*8*) nuclear splicing extracts is shown in Fig. 1.

Owing primarily to methodological difficulties involved in working with plant systems, studies of plant UsnRNAs remained for a long time, up until the mid '80s, a neglected field of plant molecular biology. In the last five years, however, most probably as a result of methods being developed for the differential extraction of UsnRNAs and UsnRNPs by the use of anticap (*54*) and anti-Sm (*49*) antibodies, and of working out adequate procedures for the isolation of plant cell nuclei particularly suited for the extraction of UsnRNAs (and UsnRNPs) in large amounts and of sufficient purity to be sequenced (*37*), an ever increasing number of reports have been

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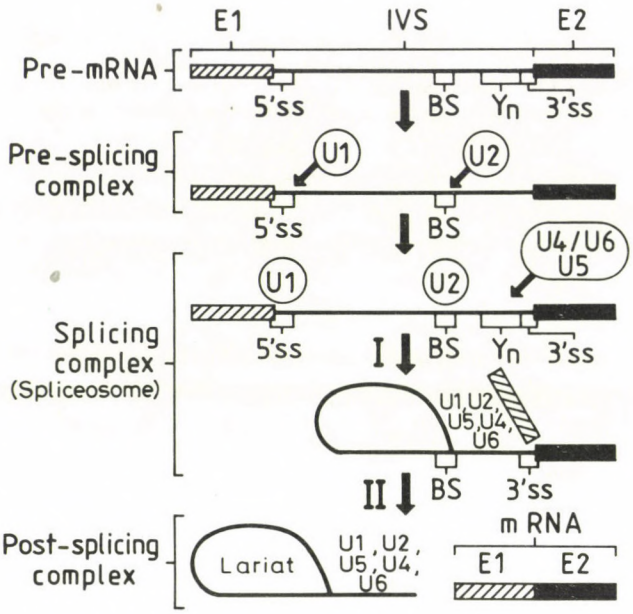


Fig. 1. *A simplified, general representation of pre-mRNA splicing.* It is assumed that all UsnRNP species remain components of the spliceosome and appear, at the end of the splicing process, associated with the intron lariat. Proteinaceous factors (hnRNP proteins, intron-binding protein (IBP), U2-auxiliary factor (U2 AF) and additional splicing factors (SF 1 to SF 4)) other than UsnRNPs (U1, U2 ....) are not considered. E1, exon 1; E2, exon 2; IVS, intervening sequence (intron); 5' ss, 5' splice site (consensus: AG↓GURAGU; ↓, cleavage site); 3' ss, 3' splice site (consensus: NYAG↓G; ↓, cleavage site); BS, branch site (consensus for vertebrates: YNYURA*Y, for the budding yeast *Saccharomyces cerevisiae*: UACUAA*C; N, any nucleotide residue, Y, pyrimidine residue; R, purine residue; *, BP (branch point)); Y_n, polypyrimidine tract where $n > 10$. The distance between the BP and the 5' end of E2 is ~ 30 nt residues on the average. I, 5' ss cleavage and lariat formation; II, 3' ss cleavage and exon ligation.

published about the structure of these molecules and of their genes (*cf. 21*) from plants, with particular emphasis on functional aspects. Since these results, interesting by themselves, usefully complement the overall picture presented by the invited speakers (C. Guthrie, W. Keller, R. Lührmann, I.W. Mattaj and J.A. Steitz) at the Symposium on "SnRNPs and Splicing of RNA" (organizers: R. Lührmann and F. Solymosy) of the 20th FEBS Meeting in Budapest, and may also affect future research trends in pre-mRNA splicing and pre-rRNA processing in general, it is felt appropriate to present a short overview of the results obtained so far with plant UsnRNAs, their genes, pseudogenes and plant UsnRNPs.

I. Plant UsnRNAs

All six major UsnRNAs (U1 to U6 RNA) have been detected in plant nuclei and have been fully sequenced (complete) either at the RNA (RNA) or at the genomic (gene) level (from some plant species partial UsnRNA sequences are also available):

U1 RNA from pea (*46*) (partial); U1 gene from common bean (*85*) (complete); U1 gene (two genes) from soybean (*87*) (complete); U1 RNA from *Chlorella saccharophila* (*40*) (complete); U1 gene (eight genes) from tomato (*1*) (complete).

U2 RNA from pea (*46*) (partial); U2 RNA (three variants) from wheat (*73*) (partial); U2 RNA from broad bean (*37*) (partial); U2 RNA from broad bean (*39*) (complete); U2 gene (six genes) from *Arabidopsis thaliana* (*82*) (5 complete and 1 partial); U2 gene from pea (*30*) (complete); U2 gene from maize (*11*) (complete).

U3 RNA from broad bean (*37*) (partial); U3 gene from tomato (*36*) (complete); U3 gene (three genes) from *Arabidopsis thaliana* (*55*) (2 complete and 1 partial); U3 gene (two genes) from tobacco (*55*) (partial).

U4 RNA (two variants) from broad bean (*41*) (complete).

U5 RNA (five variants) from pea (*46*) (complete); U5 gene from *Arabidopsis thaliana* (*84*) (complete).

U6 RNA from broad bean (*38*) (complete); U6 gene from tomato (*77*) (complete); U6 gene (three genes) from *Arabidopsis thaliana* (*89*) (complete).

It should be noted that the expression of some of the plant UsnRNA genes listed above has not been tested or the trial was unsuccessful.

A comparative survey of the sequence data on plant UsnRNAs indicates that they have the following structural features in common with their metazoan counterparts (many, but not all of these characteristics are shared also by the yeast UsnRNA equivalents):

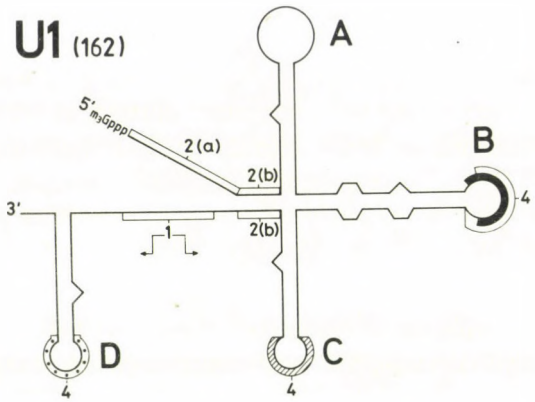
/i/ Very similar **length.**

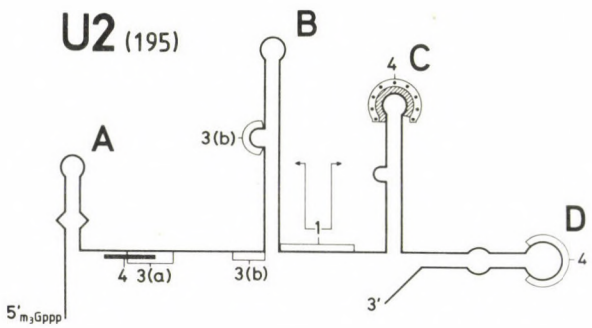
/ii/ Varying degree (~60-70% on the average) of **sequence similarity** within the individual UsnRNA species. In this respect, plant U6 RNA is phylogenetically the most conserved (>80% sequence similarity), and plant U3 and U5 RNAs are the least conserved (~50-55% sequence similarity) UsnRNA species.

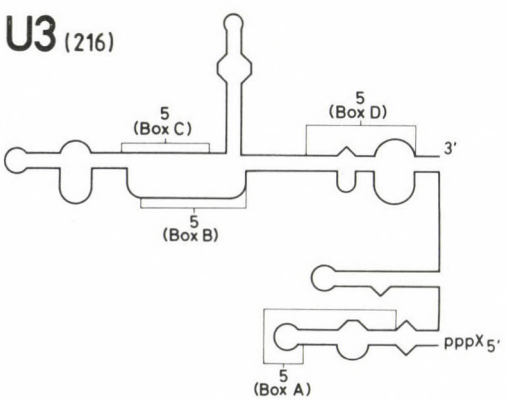
/iii/ Presence of **modified nucleosides** (mostly Ψ and sugar methylations) in the 5' half of the molecules.

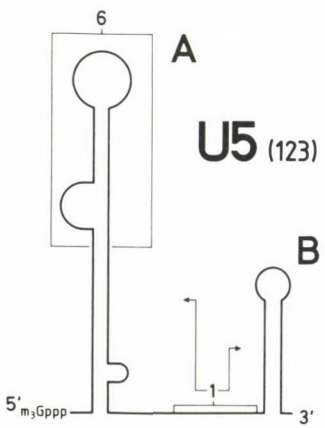
/iv/ Presence of an $m_3^{2,2,7}G$ **cap structure** at the 5' end of U1, U2, U4 and U5 RNAs. Plant U6 RNA has an as yet unidentified, unusual cap structure. In mammals, this is γ-monomethyl triphosphate (*72*). Plant U3 RNA also has an unusual cap, rather than the canonical m_3G cap structure all other U3 RNAs sequenced so far possess.

/v/ Practically identical **secondary structure** with the exception of plant U3


U1 (162)
A
B
C
D
5'm3Gppp
2(a)
2(b)
3'
1
4


U2 (195)
A
B
C
D
3(b)
4
1
4 3(a)
3(b)
5'm3Gppp
3'


U3 (216)
5 (Box C)
5 (Box D)
5 (Box B)
5 (Box A)
3'
pppX 5'


6
A
U5 (123)
B
1
5'm3Gppp
3'

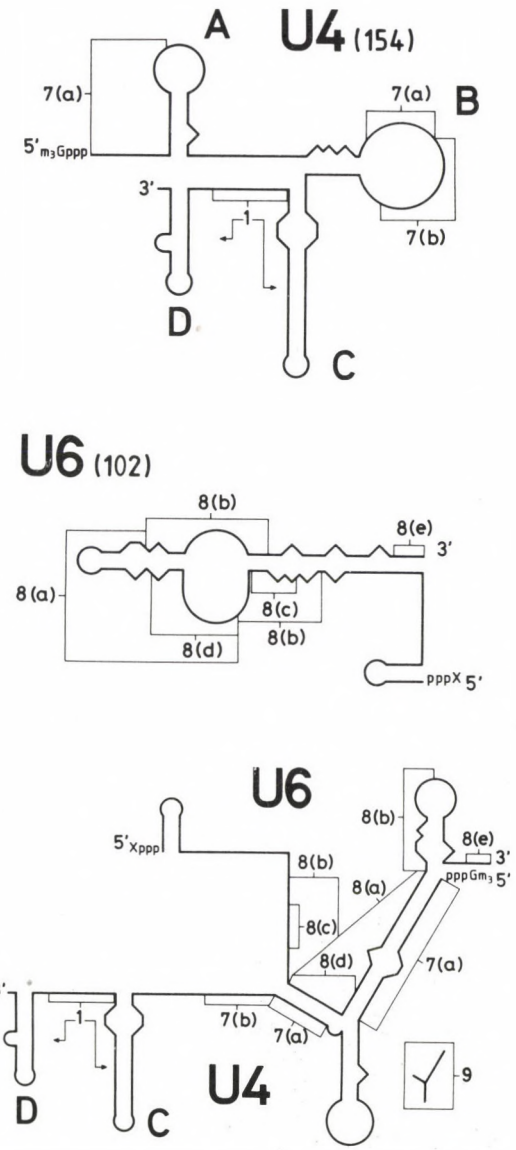


Fig. 2. *Schematic representation of the evolutionarily highly conserved secondary structures of the major UsnRNAs from plants.* Phylogenetically conserved structural elements of (possible) functional importance are denoted according to the figure and letter symbols that subdivide **Chapter I** (vii) which the reader is referred to. Terminal loops are marked by capital letters. The lengths of the individual plant UsnRNA species are given in parantheses in terms of the average number of nucleotide residues.

RNA whose 5' end portion can conveniently be folded into two stem-loops similar to U3 RNA from *Schizosaccharomyces pombe* (*67*) and unlike mammalian U3 RNA which folds into a single stem-loop structure at its 5' terminal part.

/vi/ Occurrence of **sequence variants** for U1 through U5 RNAs and absence of sequence variants for U6 RNA. A generally observed phenomenon with plant UsnRNAs is the exceptionally high abundance of sequence variants, compared to what has been found with UsnRNAs from other eukaryotes. This perception has gained ample experimental support whenever UsnRNAs from plants were analyzed either at the RNA (*20, 41, 46, 73*) or at the genomic (*1, 11, 55, 82, 87, 88*) level. By using oligonucleotides directed against conserved UsnRNA sequences in Nothern blots, Egeland et al. (*20*) reported the occurrence in monocot nuclei of size variants of U2 RNA and U4 RNA ranging from 208 to 260 and from 159 to 176 nucleotides in length, respectively.

/vii/ Existence of **evolutionarily highly conserved structural elements** (Fig. 2) of (probable) functional importance and present also in most of the non-metazoan UsnRNAs:

1. *In U1, U2, U4 and U5 RNAs*: Domain A defined as two stem-loops linked by a single-strand stretch containing the Sm antigen binding site (consensus sequence: $RAU_{4-6}GR$; *cf. 7*) which directs the binding of a common set of UsnRNP proteins reactive with the so-called anti-Sm antibodies (*cf. 49*). In some of the plant U2 and U4 RNAs one of the U residues in the above consensus sequence is replaced by a C residue. Instead of a G residue inserted in this stretch of Us in both vertebrate and plant U1 RNAs, there is a C in an algal U1 RNA.

2. *In U1 RNA:* (a) The 5' terminal domain AUACUUACCUG including the 5' splice site recognition sequence (*cf. 50, 62, 94*). (b) A "closure" (*cf. 45*) or "long range interaction" (*cf. 26*) structure which is a sequence - nonspecific motif and probably serves as a "stop signal" in the base-pairing interaction between the 5' terminal domain of U1 RNA and the 5' splice site of pre-mRNA.

3. *In U2 RNA:* The so-called "U2 domain" (*cf. 2*) which extends to about 80 nucleotide residues from the very 5' end of the molecule and encompasses both (a) the branch site recognition sequence (*cf. 65*) and (b) structural elements needed for pseudoknot formation (*cf. 3*).

4. *In U1 and U2 RNAs:* Single-stranded (mostly loop) regions, some of which are similar (▭ and ▭ in Fig. 2) and some of which are complementary (▨ and ▬ in Fig. 2) to each other. These common structural elements may enable U1 and U2 RNAs to interact with each other and/or with the same RNA or protein factor (*cf. 40*) during their functioning in pre-mRNA splicing.

5. *In U3 RNA:* Boxes A to D (*cf. 32*). The function of these evolutionarily

conserved sequence stretches is not yet clear. Box C and/or Box D are likely to interact with proteins (probably fibrillarin) specific for nucleolar snRNPs (*cf. 81*).

6. *In U5 RNA:* Loop A and part of its supporting stem. This structure is apparently not involved in RNA-RNA interaction. Its function is not known. It is interesting to note that this region of U5 RNA (from nt position 29 to nt position 48 in pea U5 RNA) shows 78% sequence similarity (*35*) to a stretch of avocado sunblotch viroid (from nt position 30 to nt position 48). No functional significance of this nucleotide sequence conservation has been revealed yet.

7. *In U4 RNA:* (a) U6 interaction domain, (*cf. 26*). Part of it (consensus sequence: UGCURRUU) is a component of Loop B. (b) The remaining part (consensus sequence: $GAAA(AC)_{0\text{-}1}\ U(A)_{0\text{-}1}\ (U/A)\ U\ (U/A)$) of Loop B has been shown by oligonucleotide-targeted degradation to be the major portion of an indispensable element needed for the *in vitro* splicing of pre-mRNA in mammalian systems (*5, 13, 43*).

8. *In U6 RNA:* (a) U4 interaction domain (*cf. 26*). (b) Two sequences inmediately abutting the U4 interaction domain and required for spliceosome assembly (*6*). (c) A short sequence (ACAGA) which has recently been proposed (*60*) to interact in yeast with the sequence UCUGU immediately downstream of the branch site recognition sequence of U2 RNA. (d) The "Box A" consensus sequence, a characteristic RNA polymerase III internal transcription signal (*cf. 15*). (e) A run of U residues at the very 3' end of the molecule, also an internal control element characteristic of RNA polymerase III transcripts (*cf. 15*).

9. *In U4 and U6 RNAs:* Plant U4 and U6 RNAs can be fitted into the so-called Y-shaped model (*9*) of interaction applicable to all U4 and U6 RNAs analyzed so far.

It should be noted that low-abundance, "minor" UsnRNAs (U7 through U14 RNAs) found in the nuclei and nucleoli of eukaryotes other than plants (*44, 61, 69, 76, 80, 81*) have not been identified yet in plant cell nuclei. Tollervey's (*78*) report on a high level of complexity of UsnRNAs in pea may have some bearing on this issue.

II. Plant UsnRNPs

The fact that plant U1, U2, U4 and U5 RNAs contain the Sm antigen binding site (see **Chapter I**, Paragraph /vii/1) strongly suggests that they are associated with the evolutionarily highly conserved Sm antigens (polypeptides B, B' and D, common to the four major spliceosomal UsnRNPs, i. e. U1, U2, U5 and U4/U6 RNPs) present in both metazoan and yeast UsnRNPs (*50, 71*). Indeed, the major spliceosomal UsnRNPs from pea nuclei could be precipitated with anti-Sm-antibodies (*79*). A detailed analysis

Table 1. *UsnRNP proteins identified in high-salt extracts from HeLa and broad bean cell nuclei.* In the HeLa system, proteins B, B', D, D', E, F and G are polypeptides common to all major spliceosomal UsnRNPs (U1, U2, U5 and U4/U6), whereas the proteins 70K, A and C are U1-specific and the proteins A' and B" are U2-specific polypeptides. The plant proteins in bold print share common epitope(s) with their mammalian counterparts.

U-snRNP proteins in				U5 RNP proteins in	
HeLa		Broad bean		HeLa	Broad bean
Letter symbol	M_r (K)	Letter symbol	M_r (K)		
-	70.0	-	54.0		
A	34.0	B/B'	**36.0**		36.0
				B/B'	
A'	33.0		**35.0**		35.0
B'	29.0	**A**	**31.0**		
B"	28.5	**B"**	**30.0**		
B	28.0	**B/B'**	**25.0**		
C	22.0	C	18.8		
		D'	17.5	D'	17.5
			17.0		17.0
D	16.0	**D**	**16.0**	D	16.0
D'	15.5				
E	12.0	E	12.5	E	12.5
F	11.0	F	11.5	F	11.5
G	9.0	G	11.0	G	11.0

of the UsnRNPs of broad bean nuclei has recently been presented by Pálfi et al (*64*). These authors, through the combined use of rabbit anti-m_3G antibodies, monoclonal antibodies, autoimmune sera with defined specificities, immunoaffinity chromatography, Mono Q anion exchange chromatography, immunoprecipitation, polyacrylamide gel electrophoresis and immunoblotting techniques, have shown that broad bean UsnRNPs containing the five major nucleoplasmic UsnRNAs U1, U2, U4, U5 and U6, are complexed with at least 13 polypeptides of approximate molecular weights of 11K, 11.5K, 12.5K, 16K, 17K, 17.5K, 18.5K, 25K, 30K, 31K, 35K, 36K and 54K. Data on the electrophoretic mobilities, combined with those on the immunospecificities of these proteins, have indicated (Table 1) that the 11, 11.5 and

12.5K proteins most probably represent equivalents of the HeLa UsnRNP core proteins G (9K), F (11K) and E(12K), respectively, the broad bean 16K and 17K proteins share structural elements with the mammalian D proteins (16K), the 17.5K protein may be a counterpart of the D' protein (15.5K) of vertebrates, the 18.5K protein is a candidate for the U1 snRNP-specific C polypeptide (22K), the 25K protein (double band) may represent variants of the common B/B' proteins of other eukaryotes, the proteins of molecular weights of 30K and 31K may be the equivalents of the U1 snRNP-specific A (34K) and the U2 snRNP-specific B" (28.5K) polypeptides, the 35K and 36K proteins would correspond to the common mammalian B/B' proteins (28/29K) and the 54K protein in broad bean is a candidate for the U1 snRNP-specific metazoan 70K protein.

III. Plant UsnRNA Genes

In view of the specific role of UsnRNPs in the cell nucleus, the genes for the UsnRNAs are expected to be transcribed very efficiently, very accurately, and in a manner independent of the transcription of mRNA, rRNA and tRNA genes.

It is not surprising therefore that, as shown in vertebrate systems, both the RNA polymerase II (Pol. II)-transcribed U1 to U5 RNA genes (*cf. 14*), and the RNA polymerase III (Pol. III)-transcribed U6 RNA genes (*cf. 47, 70*) have quite unique transcription signals (*cf. 16, 66*): /i/ the promoter and enhancer regions of U1 to U5 snRNA genes cannot be interchanged with analogous elements of mRNA genes, and /ii/ both the Pol. II-, and Pol. III-class snRNA genes appear to have some structural features in common, presumably due to the need for co-ordinate accumulation of all the spliceosomal UsnRNA species.

1. *Genes for U1, U2, (U4) and U5 RNAs*

In vertebrates, the UsnRNA genes transcribed by Pol. II (U1 to U5 RNA genes) have a proximal sequence element (PSE) at about nt position -50 and a distal sequence element (DSE) around nt position -250 in their 5' flanking region. PSE is a TATA-box equivalent (with no sequence resemblance to the canonical TATA-box whatsoever) and is responsible for the correct initiation of transcription. DSE encompassing the octamer (O) motif ATGCAAAT and Sp1 binding site is an enhancer-like element. In their 3' flanking region, around nt position +20, these genes have a loosely conserved sequence, the "3' Box", which has been shown in some cases to be required for proper 3' end formation.

In plants, the genes for U1, U2 and U5 RNAs (see references in **Chapter I**) have in their 5' flanking region the canonical TATA Box (consensus sequence: TATAAA) centered around nt position -30, which functions as the vertebrate PSE

(*83*). In addition, they have between nt positions -70 and -80, i.e. about four helical turns further upstream, a so-called upstream sequence element (USE) with no resemblance in sequence (consensus sequence: RTCCCACATCG) to the vertebrate DSE. Deletion of the USE, changing its orientation or introducing single point mutations, substantially decreased the transcription of a U2 gene in transfected protoplasts of *Nicotiana plumbaginifolia* (*83*). The 3' flanking region invariably starts with a CA dinucleotide residue which is followed, about 4 to 9 nt residues further downstream, by a 3' consensus sequence (ARTNNAA) different from the vertebrate "3' Box". Plant genes for U4 RNA have not been described yet.

2. *Genes for U6 RNA*

In vertebrates, the transcription of the genes for U6 RNA by Pol. III depends on three upstream signals: the DSE and the PSE (the same as for the UsnRNA genes transcribed by Pol. II), as well as an AT-rich box which resembles the TATA Box and is the dominant signal conferring Pol. III-specificity to the promoter (*51, 58*). No intragenic signal, although present in U6 RNA genes (see **Chapter I**, Paragraph /vii/ 8(d)) is needed for their efficient transcription (*17*).

In plants, as shown by analysis of the expression of three U6 RNA genes of *Arabidopsis thaliana* in transfected protoplasts of *Nicotiana plumbaginifolia* (*89*), the genes for U6 RNA are transcribed by Pol. III (resistance to α-amanitin). The 5' flanking region of these genes was shown to contain the same two upstream elements (the USE and a TATA-like box) as the plant UsnRNA genes transcribed by Pol. II do (only the latter element has a somewhat different nucleotide sequence), but at a reduced spacing: they were three rather than four helical turns apart. A U6 RNA gene candidate from tomato has the same structure (*77*). Using synthetic U6 genes, Waibel and Filipowicz (*89*) have demonstrated that the USE and TATA elements are indispensable for transcription, the TATA boxes of U2 and U6 genes are interchangeable, and the intragenic A Box-like sequence of the plant U6 gene is not essential for transcription, as is also the case with the metazoan U6 genes (*17*). Recently, Waibel and Filipowicz (*90*) prepared hybrid UsnRNA genes in order to investigate whether conversion of a Pol. III promoter into a Pol. II promoter, and *vice versa*, can be achieved by changing the distance between the USE and the TATA-like element. They found that insertion of 10 bp of DNA between the USE and the TATA-like box of the Pol. III U6 gene promoter changed it into an efficient Pol. II-specific promoter and, the other way round, a Pol. II promoter could be turned into a Pol. III promoter by manipulating the spacing between the USE and the TATA-like element, as long as the distance between the TATA-like sequence and the initiation site conformed to the U6 gene consensus. Transcription of the *Arabidopsis* U6 RNA genes

terminates within stretches of T residues adjacent to the coding region (*cf. 21*). The same structural element, a Pol. III termination signal, is also present in a U6 RNA gene candidate from tomato (*77*).

3. *Genes for U3 RNA*

In mammals, the genes for the non-spliceosomal, nucleolar U3 RNA are transcribed by Pol. II (*cf. 16*), similarly to those for the spliceosomal U1, U2, U4 and U5 RNAs, and their transcription signals are also similar to those of the above spliceosomal UsnRNAs. There is, however, a U3-specific motif in the mammalian genes that encode U3 RNA (*59, 93*).

Upon analyzing the upstream and downstream flanking regions of a *bona fide* gene for tomato U3 RNA, Kiss and Solymosy (*36*) postulated that *in plants*, in contrast to mammals, U3 RNA genes are transcribed by a Pol. III-type enzyme rather than by Pol. II, because /i/ in the 3' flanking region of both the tomato U3 RNA gene and a tomato U6 RNA gene candidate (*77*) the "3' consensus sequence", a possible transcription termination signal (*cf. 84*) characteristic of the plant U1, U2 and U5 genes, is absent. /ii/ Nine nt residues downstream of the 3' end of the coding sequence, there is an eight-nt-long pyrimidine block flanked by purines in both the tomato U3 RNA gene (T_8) and the gene candidate for tomato U6 RNA (TTCTTTTC). /iii/ In the 5' flanking region of both the tomato U3 RNA gene and the tomato U6 RNA gene candidate, the TATA-like element and the USE are positioned closer to each other and also to the cap site than in the genes for the plant spliceosomal UsnRNAs studied to date (for references see **Chapter I**). /iv/ Although both U3 and U6 RNAs from tomato have a cap structure at their 5' end, neither of them could be precipitated by anti-m_3G antibodies, in contrast to the plant U1, U2, U4 and U5 RNAs.

Since two U3 RNA genes from *Arabidopsis* have the same structure as the tomato U3 RNA gene, and their transcription is resistant to α-amanitin (*55*), it is safe to assume that plant genes coding for U3 RNA are transcribed by Pol. III.

The differences in transcription signals between vertebrate and plant UsnRNA genes are shown schematically in Fig. 3.

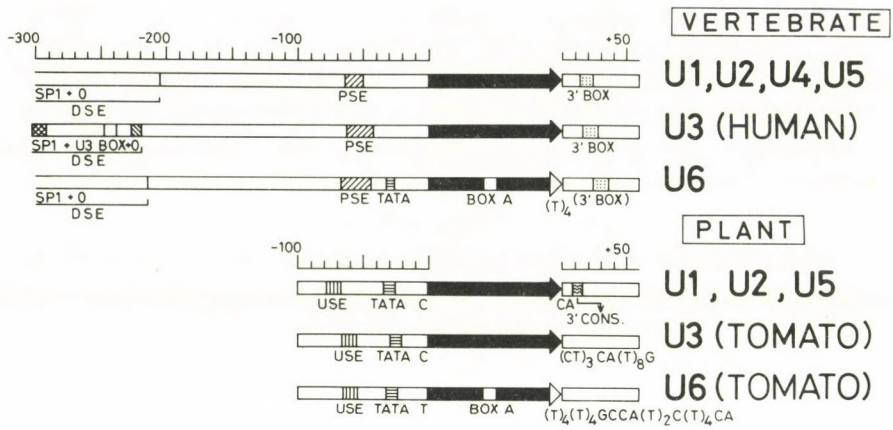


Fig. 3. *Shematic representation of the structures of UsnRNA genes from vertebrates and from plants.* For more details (including the abbreviations) see **Chapter III.**

IV. Plant UsnRNA Pseudogenes

In the genome of a number of organisms, UsnRNA-related sequences have been found to be present in multiple copies. A considerable number of these loci proved to be pseudogenes rather than *bona fide* genes (*cf. 16*). UsnRNA pseudogenes, as revealed primarily by scrutinizing the human genome (*18, 28*), fall into two main groups: those generated by RNA-mediated mechanisms, and those which have arisen through DNA-mediated events. Strangely enough, searches in plant genomes for UsnRNA-related sequences did not reveal the presence of clearly discernible pseudogenes, either for U2 and U5 RNAs in *Arabidopsis thaliana* (*82, 84*), or for U1 RNA in common bean (*85*) and soybean (*87*). Of course, the possibility cannot be ruled out that some of the plant UsnRNA gene candidates whose expression has not been tested or was unsuccessful (for references see **Chapter I**) are in fact pseudogenes with some point mutations in their coding region.

Two reports, however, conclusively indicate that in the plant genome both types of UsnRNA pseudogenes occur: in the tomato genome a U1 pseudogene generated by RNA-mediated mechanisms (*42*), and four U3 pseudogenes generated by

DNA-mediated events (*36*) have been detected. For more details the reader is referred to the original publications.

V. Concluding Remarks and Perspectives: Functional Aspects

The fact that the abundant, nucleoplasmic UsnRNAs as well as the nucleolar U3 RNA from plants possess all those evolutionarily highly conserved sequence blocks which have been shown or assumed to be of functional significance (see **Chapter I**) strongly suggests that the major plant UsnRNAs play essentially the same roles in pre-mRNA splicing and pre-rRNA processing as their equivalents in other eukaryotes. This conclusion is supported by /i/ the presence of conserved consensus sequences at both the 5' and 3' splice junctions and probably also at the branch site of plant pre-mRNAs (*cf. 10, 12*) and also by /ii/ the similarity between plants and mammals in the protein complement of the major UsnRNPs (see **Chapter II**). Thus, the very 5' end of plant U1 RNA is quite likely to base-pair with the consensus sequence at the 5' splice site, and the branch site recognition sequence (GUAGUA) of U2 RNA with a sequence block around the branch point of plant pre-mRNAs. However, apart from these two basic UsnRNA - intron interactions, more details about plant pre-mRNA splicing cannot be envisaged at this stage with any degree of certainty. The principal reason of our ignorance in this field is the lack of success, despite serious efforts in many laboratories, in the preparation of functional *in vitro* splicing systems from plant nuclei. Practically all information concerning the interplay of pre-mRNA, UsnRNPs, and a number of protein factors during splicing in multicomponent splicing complexes, the spliceosomes (*8, 22*), came from studies of *in vitro* splicing systems obtained from nuclear extracts (*19*) of yeast and HeLa cells (for a concise, up-to-date review of the subject see (*53*)).

Therefore, thorough studies of plant pre-mRNA splicing in plant *in vitro* systems would be crucial, the more so, because we have good reasons to believe that some essential factors involved in the splicing of pre-mRNAs are different in plants and mammals. Intron structure is certainly one of these factors. Substantial differences occur on the proximal side of the 3' splice junction. In mammals, this stretch of nucleotides is very rich in pyrimidine residues (conventional designation: the polypyrimidine tract), and is involved, among others, in the binding of a protein factor, U2 AF which, in turn, recruits U2 snRNP to the pre-mRNA for interaction with the branch site by base pairing (*48, 63, 92, 95*). In this region of plant introns, purine rich stretches frequently replace the prominent polypyrimidine tract of mammalian introns (*10, 12, 29*). Hanley and Schuler (*29*) divided plant introns into a purine-rich class and a pyrimidine-rich class, and found that the proportion of purine- and pyrimidine-rich

introns varies significantly in monocots and dicots. This difference in intron structure may account for the RNA processing deficiencies encountered when monocot introns were expressed in dicots (*34*). In experiments using a number of carefully designed synthetic introns, Goodall and Filipowicz (*23*) have recently provided convincing evidence in support of an earlier finding (*91*) that in plant cells a polypyrimidine tract upstream of the 3' splice site of pre-mRNA is not necessary for branch site selection, and that intron recognition by the plant splicing machinery depends absolutely on the presence of AU-rich sequences in the introns (the "AU-bias") for their splicing out to occur in transfected protoplasts of *Nicotiana plumbaginifolia*. This basic finding helps interpret the inconsistent results of earlier experiments on the splicing of plant pre-mRNAs in animal systems and *vice versa* (*4, 12, 31, 86*). It also underlines the vital importance of as yet unidentified plant protein factors in the splicing of plant pre-mRNAs. In anticipation of finding such plant-specific RNA-binding proteins Goodall et al. (*25*) have recently tried, by using PCR reactions on tobacco cDNA, to detect plant proteins harboring the RNP domain (*cf. 57*). Intron length is yet another factor probably involved in differences in splicing effiency between plant, yeast, insect and mammalian systems (*24*).

An additional aspect emerging from studies of plant UsnRNAs concerns the possible functional significance of the comparatively large number of sequence variants among UsnRNAs in plants. Recently, it has been hypothesized (*74*) that such variants may be involved in regulated and/or alternative splicing of plant pre-mRNAs. In this respect, putative transcripts of eight U1 RNA gene candidates (*1*) are of particular interest because they differ in structural elements that have been identified in vertebrate U1 RNA as sites of interaction with the U1 RNA-specific proteins 70K, A and C (see references in (*1*)). The same assumption may apply to length variants (*20*) and alleged minor UsnRNAs (*78*) observed in plant nuclei. The elegant method (*55*) of amplifying plant UsnRNA gene sequences using primers specific for an upstream promoter element and conserved intragenic regions may serve as a very useful tool for a better characterization of these elusive small RNA molecules in plants, and for discovering new ones.

The main conclusion from the work done with plant UsnRNPs (see **Chapter II**) is that the UsnRNP proteins of plants exhibit evolutionary conservation. This applies much more to antigenicity than to size, with the exception of protein D. The difference in apparent molecular weight between the vertebrate and the plant B/B' proteins is particularly remarkable and also the occurrence in broad bean of size variants of the proteins B, B' and those in the D - D' region. Whether microheterogeneity in nucleotide sequence observed in the Sm antigen binding site of plant U1, U2, and U4 RNAs (see **Chapter I**, /vii/1) mirrors heterogeneity in their protein complements

remains to be seen. A thorough characterization of plant UsnRNP proteins at the level of cDNA libraries (*cf. 52*) seems to be an absolute requirement for a better understanding of the function of these important *trans*-acting factors in plant pre-mRNA splicing. The assembly of UsnRNP particles, including the nature and processing of UsnRNA precursors, a thoroughly investigated topic with vertebrate UsnRNPs (*cf. 56, 66, 96*), has been addressed only once so far with plant UsnRNAs as experimental objects. Even in this case, the assembly of plant U1 RNAs was assayed with U1 RNA-specific Xenopus proteins in Xenopus egg extracts, i.e. in a heterologous system (*27*). The reason of this neglect may be explained again by methodological difficulties inherent in plant systems: plants do not produce Xenopus oocytes!

Structural and functional analyses of plant UsnRNA genes (see **Chapter III**) have yielded, so far, the most extraordinary results in the field, notably: /i/ the structure of UsnRNA gene promoters and the determinants of RNA polymerase specificity are completely different between vertebrates and plants, and /ii/ the genes for the nucleolar U3 RNA are transcribed in plants by Pol. III rather than by Pol. II, as in vertebrates.

As far as plant UsnRNA pseudogenes (see **Chapter IV**) are concerned, the molecular characterization of a U1 RNA pseudogene from tomato (*42*) pointed to the existence in plants of a genetic machinery supporting the reverse flow of information from RNA to DNA. The particular location of four U3 RNA pseudogenes in the immediate vicinity of a *bona fide* U3 RNA gene in the tomato genome (*36*) made it possible to deduce the chronology of events leading to the formation of these tandemly repeated pseudogenes.

In conclusion, it should be stressed once more that the development of *in vitro* splicing systems from plant nuclei seems to be vital for getting a deeper insight into the function of the major UsnRNPs in the splicing of plant pre-mRNAs and also for the enhancement of further research concerning detection and characterization of additional *cis*- and *trans*-acting factors operative during plant pre-mRNA splicing. Even before this is done, however, complementation of HeLa and/or yeast *in vitro* splicing systems, depleted of their endogenous splicing components, with proper plant equivalents (e.g. individual UsnRNPs) would be a reasonable step to take for a better understanding of the post-transcriptional regulation of gene expression in plants.

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BILIRUBIN AND MORPHINE GLUCURONIDATION IS NOT INHIBITED BY A CYCLIC-AMP MEDIATED MECHANISM IN MURINE LIVER

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SUMMARY

Several reactions of the Ist and IInd phase of biotransformation have been reported to be under a negative cyclic-AMP dependent control. Conjugation of bilirubin and glucuronidation of morphine were investigated in isolated mouse hepatocytes and in mouse hepatocyte microsomes, respectively. N^6,O^2-dibutyryl cyclic-AMP did not inhibit conjugation of bilirubin in isolated hepatocytes. ATP and the dissociated catalytic subunit of the cyclic-AMP dependent protein kinase did not influence the glucuronidation of morphine, while inhibited the formation of p-nitrophenol glucuronide in microsomes prepared from isolated mouse hepatocytes.

INTRODUCTION

Cofactor supply for the quantitatively most important processes of both the Ist and IInd phase of biotransformation in the liver, mixed function oxidation (NADPH) and glucuronidation (UDP-glucuronic acid), derives from the carbohydrate metabolism (1). It is proven that in starvation the priority of glucose production over other important processes is accomplished through a cyclic-AMP (cAMP) dependent control. In addition to regulations of these types it has been shown that the well known positive cAMP dependent regulation of gluconeogenesis is combined with a negative cAMP dependent regulation

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of several mixed function oxygenases in phase I of biotransformation (2-4). As NADPH supply for mixed function oxidation derives mainly from the intermediates of gluconeogenesis (1, 5-7) the cAMP dependent inhibition of mixed function oxidation favours the formation of glucose.

UDP-glucuronic acid supply for glucuronidation is originated from glycogenolysis (8), which is also under a positive cAMP dependent control. Conjugation with UDP-glucuronic acid is catalyzed by various UDP-glucuronosyl transferases (GT) a family of enzymes having different but frequently overlapping substrate specificities (9, 10). It has been reported that p-nitrophenol conjugation is inhibited by N^6,O^2-dibutyryl cAMP in isolated mouse and rat hepatocytes (4, 11), and also p-nitrophenol-GT activity is decreased by the dissociated catalytic subunit of the cAMP dependent protein kinase in mouse hepatocyte microsomes (4). It was tempting to assume that GT-s are in general under a cAMP dependent inhibition and this regulation also favours the formation of glucose. Therefore, it was of special interest to investigate the glucuronidation of bilirubin, one of the vital functions in hepatocytes. From this respect glucuronidation of bilirubin is catalyzed by other GT isozymes than p-nitrophenol GT (9). The conjugation of an exogenous substrate, morphine, which is a model substrate of the GT_2 isozyme (10) was also studied.

MATERIALS AND METHODS

Isolated hepatocytes were prepared from male CFLP mice (20-25 g body weight) fed ad libitum with the collagenase perfusion method and were incubated as detailed previously (12). Bilirubin glucuronidation of hepatocytes was measured according to (13). The ethyl anthranylate azopigment of conjugated bilirubin was determined by the method of Heirwegh (14). Microsomal membranes were prepared from isolated hepatocytes essentially as described by Pilkis et al. (15) as detailed previously (16). Morphine GT activity was assayed as described in (17). p-Nitrophenol GT activity was determined as reported in (9). The dissociated catalytic subunit of the cAMP dependent protein kinase was prepared from rabbit skeletal muscle as detailed earlier (16). The specific activity of the preparation was 50-120 nmole phosphate transferred·min^{-1}. mg $protein^{-1}$ when it was measured with H2b histone as substrate. 10-12 µg protein of catalytic subunit preparation/ml incubation mixture was applied as indicated. DNA content was measured by Burton (18), protein content by Lowry et al. (19).

RESULTS AND DISCUSSION

Isolated hepatocytes were incubated with 100 μM bilirubin for 30 min. The conjugation of bilirubin was examined in the presence of various concentrations of dibutyryl cAMP. The rate of bilirubin glucuronidation was constant in the course of the incubation (data not shown) and was not affected even by 10^{-3} M dibutyryl cAMP (Fig. 1).

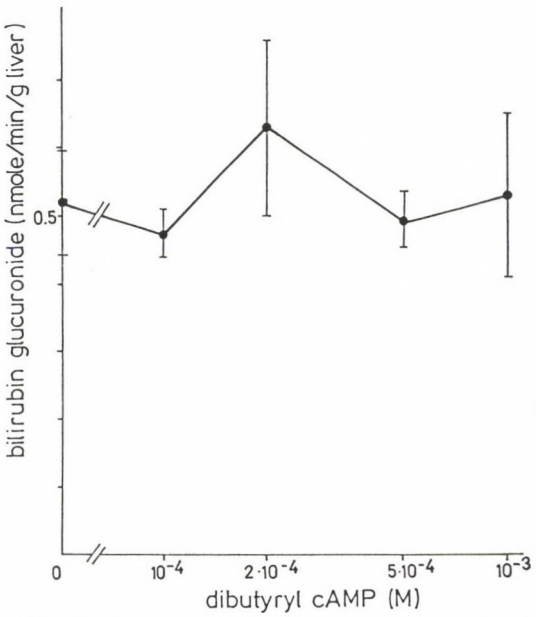

Fig. 1. Bilirubin conjugation in isolated mouse hepatocytes in the presence of dibutyryl cAMP. Isolated mouse hepatocytes were incubated with 100 μM bilirubin in the presence of various concentrations of dibutyryl cAMP for 30 min. The conjugation of bilirubin was determined. Vertical bars indicate +S.D. (n=3).

Fig. 1 demonstrates that the conjugation of bilirubin is not under a cAMP dependent negative control. It shows that in starvation the cAMP dependent control does not affect a process of vital importance the conjugation of physiological substrate, the bilirubin glucuronide formation in the IInd phase of biotransformation. It is noteworthy that in cases of mixed function oxygenases in the Ist phase of biotransformation the isozymes of the P450 IIE 1 gene subfamily, which were also not

inhibited by the cAMP dependent protein kinase (4, 20, 21) have an important physiological subatrate, the acetone (22).

On the other hand, the conjugation of an exogenic substrate, phenolphthalein could also not be inhibited this way in mouse hepatocytes or in microsomes (4). In another series of experiments the cAMP dependence of another isozyme, the GT_2, which catalyses the glucuronide conjugation of morphine was investigated. Numerous different compounds can be derived from morphine in the course of its biotransformation, although in most species morphine glucuronide is the main metabolite. For this reason the glucuronidation of morphine was investigated in a cell free system in the presence of exogenous UDP-glucuronic acid. Table 1 shows that in microsomal membranes prepared from isolated mouse hepatocytes the addition of ATP or ATP and the dissociated catalytic subunit of the cAMP dependent protein kinase did not alter the formation of morphine glucuronide but inhibited the glucuronidation of p-nitrophenol (4).

Table 1. Formation of morphine and p-nitrophenol glucuronide in the presence of the dissociated catalytic subunit of cAMP dependent protein kinase in microsomal hepatocyte membranes

Microsomal membranes were prepared from isolated mouse hepatocytes and were incubated in the presence of 1.5 mM morphine or 0.5 mM p-nitrophenol. The formation of morphine or p-nitrophenol glucuronide was determined in the presence of 5 mM ATP and the dissociated catalytic subunit of the cAMP dependent protein kinase (C). Mean ± S.D. (n=4).

Addition	morphine glucuronide	p-nitrophenol glucuronide[a]
	(μmol/mg protein/min)	
none	2.803 ± 0.443	22.82 ± 2.08
ATP	2.844 ± 0.358	17.03 ± 2.03
ATP + C	3.002 ± 0.172	13.68 ± 1.39
C	3.052 ± 0.294	20.27 ± 1.58

[a]Data taken from Ref. 4.

It is concluded that similarly to phenolphthalein-GT, bilirubin- and morphine-GT were not influenced by a cAMP dependent regulation. The capacity of different GT-s is also different and depends on the species investigated as well. The capacity of p-nitrophenol GT is higher than that of phenolphthalein-, morphine- or bilirubin-GT. Thus, it can be assumed that primarily the high capacity GT-s are under a cAMP dependent regulation, while the low capacity isozymes are not. However, it can also be supposed that p-nitrophenol GT engaged in the metabolism of planar phenols is an exemption, and in general glucuronidation is not under cAMP dependent control.

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EFFECT OF A REMOTE CHIRAL CARBON ATOM IN THE SUBSTRATE ON THE STEREOSPECIFICITY OF AMINOACYLASE

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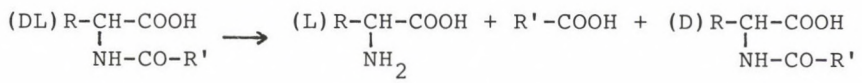
INTRODUCTION

Aminoacylase (E.C.3.5.1.14) catalyzes the following process:



with high substrate specificity (1-5). The mechanism of the reaction has already been described (6). It has been concluded that $K_{m,app} = K_S$ and a nucleophyl attack occurs on the carbon atom of the acylamino group and the rate determining step is the decomposition of the ES complex. The mechanism as well as a free energy relationship (7) allows interpreting the stereochemistry of the reaction. This free energy relationship explains and describes quantitatively the stereospecific hydrolysis of substrates having a chiral carbon atom in alpha position of the acyl moiety. A substituent in alpha position of the acyl part has a decisive effect on the stereospecificity. These results obtained with α-acyl-amino acids were earlier explained quantitatively. No study has, however, been made on the effect of an asymmetric centre in the acyl part far from the reaction centre.

We present in this paper that there is a marked difference in the reaction of diastereomer acyl-amino acid substrates posessing an asymmetric carbon atom in the acyl moiety

in γ-position. This high stereoselectivity can be interpreted by a fixed conformation of the acyl moiety in the enzym-substrate complex.

MATERIALS AND METHODS

All substrates were prepared in our laboratory by acylating amino acids in a usual reaction with acid chlorides or anhydrides by the Schotten-Baumann method. All materials were purified to a constant melting point and optical rotation. Purity was checked by elemental analysis, IR and NMR spectroscopy.

Table 1. Physical constants of Aminoacylase I substrates

Substrate	Melting point °C	$\lvert\alpha\rvert_D^{25}$ (c=3%; ethanol)
n-valeryl-N-norvaline	64 - 65	- 7.4
n-caproyl-L-norvaline	86 - 87	-14.9
(R,S)γ-methylcaproyl--L-norvaline	98 -102	-11.4
(R,S)γ-phenylvaleryl--L-alanine	49 - 54	-16.3
(S)-(+)-γ-methylcaproyl--L-norvaline	105 -107	- 5.1

The (R,S)-γ-methylcaproyl-L-norvaline and the (R,S)-γ-phenylvaleryl-L-alanine substrates were not recrystallized to avoid the fractionation of diastereomers in the synthesis. To ascertain the relative proportion in the mixture, each of the diastereoisomer substrates were hydrolyzed with 5 N HCl and the liberated acids were isolated. The lack of any optical rotation of the liberated γ-methyl-caproic and γ-phenylvaleric acids indicated the absence of fractionation of diastereomers in course of the synthesis. The diastereomeric mixtures, (R,S)-γ-methylcaproyl-L-norvaline and (R,S)-γ-phenylvaleryl-L-alanine, were therefore a 1:1 mixture of the diastereomeric components.

Enzyme assay. The activity of aminoacylase I (purchased from SIGMA Chem. Comp., St. Louis, Mo. USA) was determined according to Bruns and Schulze (10). The enzymatic cleavage rate of N-acyl parts was determined by measuring the change in the absorbance according to Rosen (11). The apparent K_m and V_{max} values were determined from Lineweaver-Burk plots and the k_{cat} values were calculated from the relationship $k_{cat}=V_{max}/E$.

Enzymatic hydrolysis of (R,S)-γ-methylcaproyl-L-norvaline. limolar aqueous solution of (R,S)-γ-methylcaproyl-L-norvaline (pH 7.2) was incubated at 38°C in the presence of

100 mg/L aminoacylase I for 18 hours, and aliquots were withdrawn and analyzed. As the enzymatic hydrolysis reached 30% of theoretical conversion, the reaction was stopped by acidic precipitation and subsequent removal of the denaturated hydrolase. Then, after weak alkalization the solution was evaporated in vacuum to the two - three hundredth of the original volume. The etheric extraction of the re-acidified (pH 1.8) reaction mixture yielded a solution containing the liberated γ-methylcaproic acid as well as the intact substrate. The separation of these compounds was carried out by petroleum ether extraction. Fractioned destillation yielded pure γ-methylcaproic acid.

Enzymatic hydrolysis of (R,S)-γ-phenylvaleryl-L-alanine. It was performed similarly to that described above. The fractioned destillation of crude γ-phenylvaleric acid yielded a pure product.

RESULTS AND DISCUSSION

Data summarized in the Table 2 suggest a high enzymatic stereoselectivity: in the case of (R,S)-γ-methylcaproyl-L-norvaline substrate the aminoacylase I prefers the compound of "R" configuration, while that from the (R,S)-γ-phenylvaleryl-L-alanine the isomer of "S" absolute configuration was favourable. The absolute configuration of optically active acids mentioned above are known.

Table 2. Products of enzymatic hydrolysis of (R,S)-γ-methylcaproyl-L-norvaline and (R,S)-γ-phenylvaleryl-L-alanine

Substrate	Conversion %	Isolated acid	Optical rotation $[\alpha]_D^{22}$	Optical purity %
(R,S)-γ-methylcaproyl-L-Nva	30	(R)-(-)-γ-methylcaproic acid	- 8.64°	71[a]
(R,S)-γ-phenylvaleryl-L-Ala	15	(S)-(+)-γ-phenylvaleric acid	+20.3°	92[b]

[a]Based on $[\alpha]^{25}$=+12.18° for pure (S)-(+)-γ-methylcaproic acid (12)

[b]Based on $[\alpha]_D$=-22.10° for pure (R)-(-)-γ-phenylvaleric acid (13)

Stereospecificity of enzymatic reaction (aminoacylase, chymotrypsin) for substrate containing a chiral carbon atom in α-position of the acyl moiety has been explained earlier (13, 14). The interpretation of the stereospecificity was based on the reversed position of two substituents connected with the chiral α-carbon atom of a substrate in the E-S complex. Different hydrolytic rates of an enzymatic reaction are due to the different bulk of the R^1 or R^2 group. A small R^2 substituent hinders the catalytic process much less than a bulkier R^1 one. A quantitative assessment for the stereoselectivity can be given by the difference in the van der Waals radii of the R groups.

Supposing that

1. the steric hindrance is due to substituents on the chiral atom being far from the optically active centre in the acyl part, thus they can approach the reaction centre and
2. substituents in reversed positions may cause different steric inhibition

we can offer a similar explanation for stereospecificity of substrates containing a chiral carbon atom in the acyl moiety far from the carbonyl group.

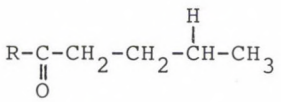
A strict analysis of kinetic constants of the two stereoisomers of γ-methylcaproyl-L-norvaline (III., IV.) and those of the n-valeryl- (I) and isocaproyl-L-norvaline (II) can solve this problem (see Table 3).

Considering the binding constants for compound I and II that are closely equal, these substrates are fixed in the ES complex by a similar strength.

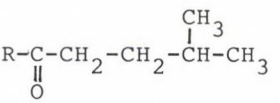
The great difference in k_{cat} values may be due to the different steric hindrances caused either by a methyl group (compound II) or by a H atom (compound I) in γ-position. The only explanation is that the alkyl chain is in a "twisted" conformation and the methyl group of the iso-caproyl compound approaches the carbonyl group, thus it reduces the possibility of the reaction between the enzyme and the substrate. Similar steric hindrance may exists for the "R" isomer of γ-methyl-caproyl-L-norvaline.

Table 3. Kinetic constants of substrates I-IV.

Substrate	$K_{m(app)}$ mM	k_{cat} s^{-1}	$k_{cat}/K_{m(app)}$ $mM^{-1}s^{-1}$
n-valeryl-L-norvaline (I)	0.40±0.06	161±13	402.0
i-caproyl-L-norvaline (II)	0.55±0.15	19.2±2.3	34.9
(R,S)-γ-methylcaproyl-L-norvaline (III)	0.35±0.06	15.2±1.2	43.4
(S)-(+)-γ-methylcaproyl-L-norvaline (IV)	0.30±0.04	2.4±0.2	8.0



I.



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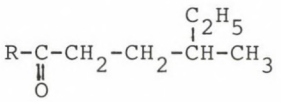

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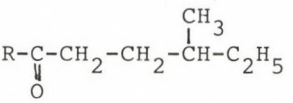

IV.

R = L-norvaline

We have no information on the kinetic constants of "R" isomers. The low reactivity of the "S" isomer and the fact that initial rates have been measured, there isn't any great difference between the k_{cat} and $k_{cat}/K_{m(app)}$ values of the "RS" and "R" compounds. The terminal ethyl group does not exert substantial steric hindrance on the reaction centre. Related to the "R" isomer, the methyl and ethyl groups are in opposite positions in the "S" isomer. The ethyl group causes higher steric hindrance than the methyl part and the reactivity of the "S" isomer is decreased. The stereoselectivity is

due to this difference. All these facts lead to stereoselectivity.

The hydrolytic behaviour of (R,S)-γ-phenylvaleryl-L-alanine in enzymatic process is similar to that of the γ-methylcaproyl derivative.

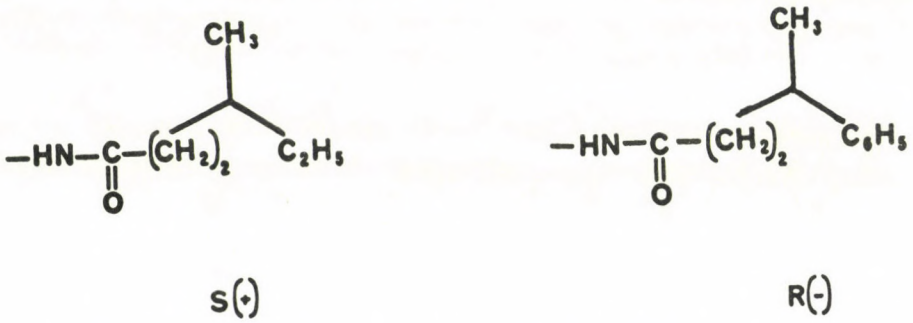

Fig. 1. Structure of (R,S)-γ-phenylvaleryl-L-alanine and (S)-γ-methylcaproyl-L-norvaline as bound to the active site of aminoacylase

Figure 1 presents the conformation of (S)-γ-methylcaproyl-L-norvaline in the ES complex which is equal to the (R)-γ-phenylvaleryl-L-alanine. This presumption can be explained by the reversed stereospecificity.

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REC MUTANTS OF ESCHERICHIA COLI DEFICIENT IN SUBUNITS OF REC BC (D) COMPLEX

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SUMMARY

The inactivation of rec BC (D) DNase upon chromatography on DEAE-cellulose was observed. Simultaneously DNA-stimulated ATPases (I and II) and DNase activities on single- and double-stranded DNA substrates were measured in Escherichia coli rec^+ and rec^- cell extracts. Normal levels of ATPase I and II were detected in rec^+ cells. Rec A^- cells were lacking DNA dependent ATPase I, while rec B single and rec BC double mutants were defective in DNA dependent ATPase II, the second major enzyme of this type. Rec B and C mutations did not change DNase activities. Rec A mutation significantly increased DNase activity on linear single-stranded substrate.

INTRODUCTION

Exonuclease V (E.C.3.1.11.5.) the ATP-dependent DNase of Escherichia coli also referred to as BC enzyme possesses multiple enzymatic activities in vitro and plays an important role in genetic recombination, repair and removal of foreign DNA in vivo (1). In vitro activities include ATP-dependent exo-, endonuclease, helicase and DNA-dependent ATPase activities.

The molecular mechanism of exonuclease V complex involves binding to the termini of DNA duplexes, unwinding of the double helical structure by the formation of a single-stranded loop and displacing the other strand (2, 3). Both 3'- and 5'-ended tails are generated with equal frequency during unwinding. The strand separation process requires ATP and is followed by cleavage (4), occuring preferentially at recombinogenic Chi sites (5, 6).

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Exonuclease V protein complex was originally thought to be encoded by rec B and rec C genes (7, 8). The cooperative manner of individual enzymes being part of the above mentioned wide range of catalytic activities is still poorly understood. Liebermann and Oishi were the first to think of the dissociable character of the rec BC enzyme complex distinguishing α and β fractions (9). α turned out to be a protein encoded by rec D gene located between thy A and arg A in the neighbourhood of rec B gene in the order of thy A- rec C- ptr- rec B- rec D- arg A (10, 11). Meanwhile the sequence of rec B (12) and rec C (13) genes have been determined. These genes have been cloned and their products amplified and purified (14, 15). Although our understanding of the rec BCD enzyme complex is still not clear the current knowledge has been reviewed by Smith (16, 17) and Lederberg (18).

The cooperative manner of dissociated ATPase and DNase activities for exonuclease V suggested a multifunctional co-operation of individual enzymes rather than a single one with multifunctional properties (19). To test the validity of this idea we addressed the question whether rec mutants are deficient in individual enzymatic activities after dissociation by DEAE-cellulose chromatography. For this purpose DNase activities on single-, and double-stranded DNA and ATPase activities dependent on DNA cofactors were traced in cell extracts of rec mutants. We describe that the major DNA-dependent ATPase, referred to as ATPase I is encoded for by the rec A gene and that rec B is the structural gene of ATPase II, the second major unwinding ATPase of E. coli. Cooperation between rec A protein and that of DNase is discussed.

MATERIALS AND METHODS

Buffers. Buffer A contained 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 2 mM 2-mercapto-ethanol, buffer B: buffer A plus 30% (v/v) glycerol. Saline-sodium citrate buffer contained 150 mM NaCl, 15 mM sodium citrate (pH 7.0).

Bacteria. Bacterial strains are described in Table 1. Cells were grown at 37°C in a Gallenkamp rotary incubator in 6 l. of Penassay broth (Difco). Bacteria were harvested in exponential phase $3\text{-}5\times10^8$ cells/ml and stored at -20°C until further processing.

Table 1. Strains of Escherichia coli K-12

Strains	Rec mutation	Other markers	Source
JC 5029	rec^+	Hfr, thi-1, ilv-318, thr-300, rel A1, spc-300	Clark, A.J.
JC 5412	rec B_{21}	Hfr, thr-300, sbcA8, rel A1, ilv-318, thi-1, rpsE2300	Clark, A.J.
KL 169	rec C_{22}	Hfr, thi-1, rel A1, deoB13,	Low, K.B.
JC 5088	rec A_{56}	Hfr, thi-1, ilv-318, thr-300, rel A1, spc-300	Clark, A.J.
JC 5491	rec B_{21} rec C_{22}	Hfr, thi-1, ilv-318, rel A1, spc-300	Clark, A.J.
594	rec^+	F^-, galK2, galT22, rpsL179	Smith, G.R.
V 314	rec A_{56}	as 594, srl300:Tn10	Smith, G.R.
V 370	rec A_{56} rec D_{1009}	as V 314	Smith, G.R.
V 371	rec A_{56} rec C_{1010}	as V 314	Smith, G.R.

The strains JC 5029, JC 5412, KL 169, JC 5088, JC 5491 were from B. Bachmann (E. coli Genetic Stock Center, USA), 594, V 314, V 370, V 371 were donated by G.R. Smith (Fred Hutchinson Cancer Research Center, USA).

Materials and radioactive compounds. /γ-^{32}P/ATP (370 GBq/mmol) was the product of Isinta (Budapest). /^{3}H/DNA from B. cereus 130 thy - was isolated as in (20). Heat denaturation of DNA was carried out at 100°C for 10 min in saline-sodium citrate buffer followed by rapid chilling in ice. DNA-dependent ATPase and ATP-dependent DNase assays on native and denatured DNA were as described (21). Protein was determined by the method of Lowry et al. (22). Purification steps of the ATP-dependent DNase from E. coli strains were the same as in (21).

RESULTS

Three DNA-dependent ATPase and a DNase specific for single-stranded DNA have been dissociated by DEAE-cellulose chromatography of E. coli cell extracts (19). The ATPases (I, II and III) eluted at 0.18, 0.24 and 0.37 M NaCl concentrations, respectively. In the present investigations elution conditions were restricted to the isolation of DNA-dependent ATPase I and II.

The distribution of ATPase I and II after DEAE-cellulose chromatography of cell extracts from E. coli rec^{+} strains is represented by Fig. 1a, ATPase I being eluted at 0.16 and ATPase II at 0.21 M NaCl concentrations. Significantly different elution profile of ATPase distribution was observed in rec A^{-} strains (Fig. 1b), with ATPase I peak missing. Similar elution profiles were observed in rec AD and rec AC double mutants (results not presented). The absence of ATPase II peak in rec B single and rec BC mutants (Fig. 1c and d) is the reflection of rec B gene being the structural gene of ATPase II. There were no considerable differences among the chromatographic profiles of ATPase activities in rec C^{-} mutants and in their parent strains (Fig. 1e).

The qualitative picture i.e. the comparison of the chromatographic profiles of DNase activities upon DEAE-cellulose chromatography of cellular extracts from rec mutants did not show significant alterations (not shown). However, the precipitability of ssDNase by ammoniumsulfate was significantly affected by the rec A^{-} mutation. While in rec^{+} strains roughly 50% of DNases were precipitated at 40% ammoniumsulfate saturation, in rec A^{-} mutants significantly less DNase was found in

the 40% ammoniumsulfate precipitate and their activities were much higher than those of the rec^+ strains (Table 2). The distribution and quantity of DNases of rec B and rec C mutants after ammoniumsulfate treatment was similar to that of rec^+ extracts (results not shown).

Table 2. DNase activities of cellular extracts from rec^+ and rec^- strains

Strain	Rec mutation	ssDNase activity of		Total
		40% $(NH_4)_2SO_4$ precipitate	60% $(NH_4)_2SO_4$ precipitate	
		%	%	%
594	rec^+	46	54	100
V 314	rec A_{56}	2	160	162
V 371	rec A_{56} rec C_{1010}	10	133	143
V 370	rec A_{56} rec D_{1009}	26	251	277

Results are given as percentages of the total DNase activity of rec^+ precipitates. Cellular extracts were made from $5x10^9$ cells, each.

DISCUSSION

The multifunctional properties (1, 23, 24) and the dissociable character of rec BC nuclease (8, 9, 15) have notoriously puzzled specialists in the field of bacterial recombination leading to suggestions which prefer the cooperation of enzymes involved in a multistep, timed process rather than a single catalytic event. Retrospectively, the inactivation of ATP-dependent DNase activity observed by several authors during DEAE-cellulose chromatography (23, 25, 26) can be explained on the basis of dissociation of independent units of a multienzyme system loosely associated by ionic forces and by attachment to DNA.

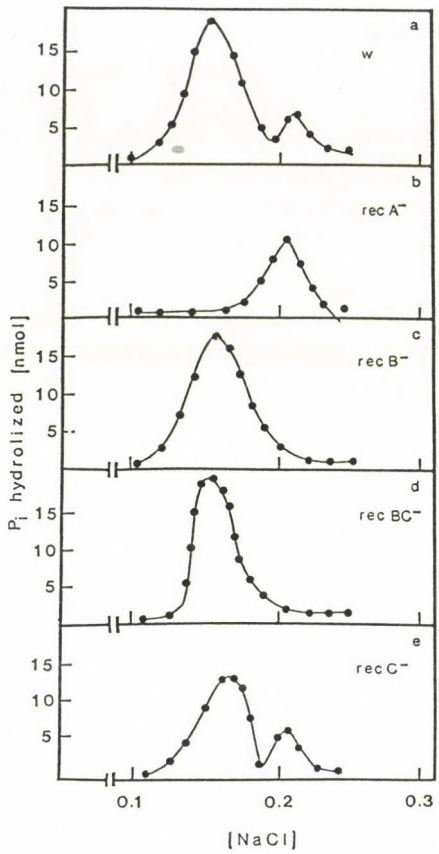

Fig. 1. Effect of rec mutation on DNA-dependent ATPase activity.
Cellular extracts were made from $5x10^9$ cells and were precipitated by partial saturation (40% and 60%) with $(NH_4)_2SO_4$. The 60% precipitate was dissolved in buffer B, dialyzed against the same buffer and applied to a DEAE-cellulose column (1x4 cm) equilibrated with buffer B. After washing with buffer B a linear gradient of 0.1-0.3 M NaCl in buffer B (160 ml total) was applied, 2 ml fractions were collected and their NaCl content was determined. DNA-dependent ATPase profiles of chromatographic fractions from a) rec^+ (JC 5029), b) rec A^- (JC 5088), c) rec B^- (JC 5412), d) rec BC^- (JC 5491), e) rec C^- (KL 169) $(NH_4)_2SO_4$ precipitates. Results are expressed as the difference of DNA-dependent and independent ATPase activities. Measurements of DNA-dependent ATPase activities were described (21).

Taking adventage of the chromatographic inactivation of ATP-dependent DNase activity and consequently the separation of ATP-phosphohydrolases with unwinding properties (19, 21) present analysis revealed that the major DNA stimulated ATPase of E. coli is encoded by the rec A gene. Rec B turned out to be the structural gene for the second major DNA stimulated ATPase earlier referred to as ATPase II (21).

The fact that rec C mutation did neither influence DNA-dependent ATPase nor ds and ssDNase activities is to some extent perplexing. In accordance with this observation Hichson et al. (15) found that rec C protein alone has neither ATPase nor exonuclease activity. Thus no specific activity can be attributed to rec C gene product suggesting a yet undefined regulatory function for the rec C gene in the ATP-dependent DNAse activity and consequently in the general recombination of E. coli. Similar cooperative manner for the rec D gene product would explain why this mutation did neither effect ATPase nor DNase activities. However, the lack of evidence of a missing or diminished DNase activity in itself does not exclude the possibility of rec C and/or rec D genes being involved in DNA cleavage. It should be mentioned that contrary to the distinct elution profiles of ATPases the separation of DNases was not as straighforward during DEAE-cellulose chromatography. One explanation for this observation might be the ionic conditions unfavourable for the separation of different DNases. Alternatively, the number of DNases in E. coli might be much less than expected.

The cooperative manner of independent ATPase and DNase units manifesting as "ATP-dependent DNase" activity does not seem to be a unique feature. Rec A mutation is known as a "reckless" mutation due to its elevated DNA degradation. Our data confirm that in rec A mutants DNase acitivity is indeed increased. Thus rec A gene product seems to control the DNase activity essential for general recombination (27).

The cooperation of rec gene products is further complicated by the discovery of rec D gene and its product (11). A concensus DNA sequence specific for ATPase binding proteins

was identified in rec D gene (28). However, we did not find correlation between rec D mutation and ATPase activity. Finch et al. (13) suggest that rec B and rec D genes might be related similarly to uvr A and uvr B genes in the uvr ABC nucleolytic activity. Uvr A is a DNA-independent ATPase which is stimulated by the product of uvr B gene through its binding to DNA in an ATP-dependent manner. As a further analogy the relationship between uvr C gene product and that of uvr AB can be mentioned. Rec C protein could function similarly in cooperation with rec BD complex.

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ENZYMATIC PROPERTIES, METAL COMPOSITION AND SH-GROUP REACTIVITY OF FRAGMENTED SARCOPLASMIC RETICULUM ISOLATED FROM RABBIT SCELETAL MUSCLE

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SUMMARY

Comparation of properties of fragmented sarcoplasmic reticulum samples isolated by several methods is reported. It was found that the protein composition does not differ significantly in samples which were or were not washed with 0.6 M KCl when isolated. In the case of samples washed with KCl solution the Zn concentration, the Ca/Mg ratio (determined from experimental data), acetylcholinesterase and superoxide dismutase activities were higher whereas Ca+Mg-activated ATPase and p-nitrophenylphosphatase activities were lower than those of samples which were not washed with 0.6 M KCl. In the latter samples the amount of SH-groups and the reactivity of fast SH-s are higher in Ca+EGTA containing media than in media containing only EGTA. In contrast in the case of samples washed with KCl solution the results are the opposite. In conclusion, washing of FSR with 0.6 M KCl alters the metal composition, enzymatic properties, SH-group reactivity and as a result of these probably the conformation of the protein samples, as well.

Abbreviations: FSR, fragmented sarcoplasmic reticulum; PNPPase, para-nitrophenyl phosphatase; ACEase, acetylcholinesterase; SOD, superoxide dismutase; DTNB, 5,5'-dithiobis (2-nitro-benzoic acid); EGTA, ethylene glicol-bis (2-aminoethyl-ether)-N,N,N',N'-tetraacetic acid; SDS PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; AASI, atom-absorption spectrometer.

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INTRODUCTION

One of the important functions of muscle sarcoplasmic reticulum (SR) is to control the contraction as well as the relaxation of muscle by altering the free Ca-concentration in the intermyofibrillar space. Processes leading to relaxation, i.e. the mechanism of Ca-influx of SR were thoroughly examined (10, 17, 31, 41). Nevertheless, processes leading to muscle contraction, i.e. the mechanism of Ca-release from SR into the intermyofibrillar space are scarcely understood (12). It is known that depolarisation of transverse tubular system initiates the release of Ca stored in terminal cysterns of the SR (7, 10, 13). To date several theories have been proposed for the mechanism of interaction between depolarisation and Ca-liberation from the SR (11, 18, 20, 26, 33, 34, 48).

To investigate the mechanism of Ca-release and influx we isolated functionally intact structures of SR. It was necessary to study the corresponding analitical and separation procedures, too. Therefore, we isolated fragmented sarcoplasmic reticulum from rabbit sceletal muscle by three various methods and examined their protein composition, metal ion concentration, enzyme kinetic properties and SH-group reactivity. This latter was particularly important because in the light of recent results (1, 4, 44) the state of some critical SH-group(s) plays important role in the control of Ca-channel activity (4) in the increasing of Ca-liberation (1, 44).

MATERIALS AND METHODS

Isolation of fragmented sarcoplasmic reticulum. The FSR samples were isolated by three various methods from rabbit sceletal muscle. 1) Suko and Hasselbach (36): Homogenization and preparation were carried out in a solution of 0.25 mol/l sucrose and 10 mmol/l TRIS-maleate, pH 7.0. To remove the myofibrillar proteins the FSR was washed with a solution of 0.25 mol/l sucrose, 0.6 mol/l KCl and 10 mmol/l TRIS-maleate, pH 7.0. (The sample was marked as FSR-S.) 2) Nakamura et al. (29): The homogenization and preparation was carried out in a solution of 100 mmol/l KCl and 10 mmol/l imidazole, pH 7.4. The myofibrillar proteins were removed with a 0.6 mol/l KCl and 10 mmol/l imidazole, pH 7.4, solution. (Marked as FSR-N.) 3) Caswell et al. (5): The samples were homogenized and prepared in a 0.25 mol/l sucrose, 1 mmol/l EDTA and 10 mmol/l

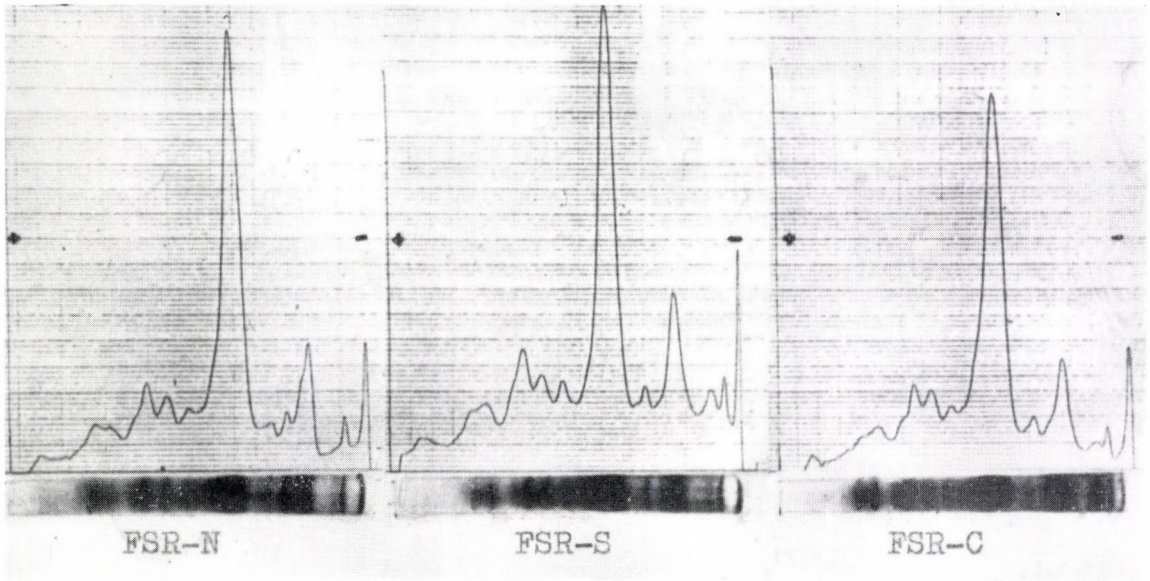

Fig. 1. SDS-PAGE gel pictures and densitograms of FSR-N, FSR-S and FSR-C isolated by the methods of Nakamura et al. (29), Suko and Hasselbach (36) and Casswell et al. (5). Details can be found in "Materials and Methods"

TRIS-maleate, pH 7.0, solution. The crude FSR was not washed with 0.6 mol/l KCl. (Marked as FSR-C.) For comparative experiments a part of the samples was washed with a solution of 0.6 mol/l KCl, 10 mmol/l TRIS-maleate and 0.25 mol/l sucrose. (Marked as FSR-Cw.)

The samples were stored in a buffer of 0.25 mol/l sucrose and 10 mmol/l TRIS-maleate, pH 7.0, at a concentration of 15-20 mg protein/ml at -30°C or -70°C.

Measurement of ATPase activity. The composition of the ATP-containing incubation mixture was: 100 mmol/l KCl, 20 mmol/l TRIS-maleate, pH 7.0, $MgCl_2$, K-oxalate and ATP 5 mmol/l, 0.5 mmol/l EGTA, with or without 0.5 mmol/l $CaCl_2$ and 30-40 µg FSR. The volume of the incubation mixture was 1 ml. The enzymatic reaction was initiated by the addition of ATP after 5 min preincubation at 25°C. In each minute 100 µl aliquots were taken from the incubation mixture and, to inactivate and solubilise the ATPase, they were placed into 0.1 ml 50 g/l SDS solution. The amount of the liberated inorganic phosphate was determined by the method of Taussky and Shorr (43).

Measurement of para-nitrophenylphosphatase activity. The composition of the incubation solution was the same as that of ATPase measurement but it contained 5 mmol/l para-nitrophenylphosphate instead of ATP. The liberation of p-nitrophenol was detected at 420 nm at 25°C. The degree of hydrolysis of p-nitrophenylphosphate was calculated with the help of $\varepsilon = 5780$ molar extinction coefficient, as determined in our laboratory.

Measurement of Acetylcholinesterase activity. The composition of the incubation mixture was the same as in the case of Ca + Mg-dependent ATPase activity determination but it contained 1.5 mmol/l acetylthiocholine iodide and 1 mmol/l DTNB rather than 5 mmol/l ATP. The degree of liberation of thiocholine was detected with DTNB at 412 nm at 25°C. The enzyme activity was calculated from the rise of the reaction curve. We used $\varepsilon = 12\ 670$ molar extinction coefficient determined in our laboratory.

Superoxide dismutase activity. We used the method of Misra and Fridovich (27) at 25°C. The incubation volume was 1 ml that contained 100-200 µg FSR protein.

Determination of calcium, magnesium and zinc concentration. It was carried out with an AASI atomabsorption spectrometer (Carl Zeiss, Jena). For the measurements the FSR samples were diluted to 0.5-2.0 mg protein/ml with a 0.25 mol/l sucrose solution. Calcium, magnesium and zinc standard solutions necessary to evaluate our findings were prepared with a 0.25 mol/l sucrose solution.

SDS polyacrylamide gel electrophoresis (SDS PAGE). Samples were prepared as described earlier (39). The electrophoresis was carried out by the method of Weber and Osborn (49) but in a 50 g/l acrylamide instead of 70 g/l. After dying and washing the gels the proportion of components in percentage was determined on the polyacrylamide gels with a

densitometer made from an Opton PM 2DL spectrophotometer, a Radelkis (Budapest) recorder (type OH-814), a home-made gel-container tube and gel-pusher motor.

Determination of SH-group content. The SH-concentration of the FSR samples was determined in the following media: 1) 100 mmol/l KCl, 20 mmol/l TRIS-maleate, pH 7.0, $MgCl_2$, K-oxalate 5 mmol/l, 0.5 mmol/l EGTA, 0.5 mmol/l $CaCl_2$, 1 mmol/l DTNB, referred below as Ca + EGTA containing incubation medium. 2) It is the same medium as No 1. but without $CaCl_2$, i.e. EGTA containing incubation medium. According to our results on AASI this "in principle" Ca-free incubation mixture contained 32 µmol/l calcium as a result of Ca-impurities in our chemicals. The volume of the incubation medium was 0.5 ml which contained 40-100 µg protein. The measurements were carried out at 412 nm at 22°C. The samples and controls were mixed before the determination of light-absorption. DTNB was applied in a 50-100 fold excess compared to the concentration of reactive SH-groups. The molar extinction coefficients (ε) were the following: Ca + EGTA containing incubating medium: ε = 12 660; EGTA containing incubating medium: ε = 13 630.

Materials. EGTA was the product of Serva (Heidelberg, FRG). DTNB and adrenaline were supplied by Sigma (St. Louis, USA). Acrylamide, bisacrylamide, SDS, bromophenolblue and Coomassie brillant blue R = 250 were the products of Shandon Southern (Runcorn, England). Mercaptoethanol and sucrose were obtained from Merck (Darmstadt, FRG) and acetylthiocholine iodide from Fluka (Buchs, Switzerland). All of the other chemicals were analitical grade from Reanal (Budapest, Hungary).

RESULTS

Gel pictures and densitometric curves obtained after SDS PAGE of FSR samples (FSR-S, FSR-N, FSR-C) are shown on Fig. 1. The main component (64-68%) of the samples is the Ca-pump protein 100-105 kD, Table 1. The most important protein components are the following: 45 kD calsequestrine, 5.6-6.6%; 55 kD high-affinity Ca-binding protein, 3.8-4.9%; 32 kD 2.1-2.5%.

There are 5-7 other distiguishable bands, as well: 340-370 kD (1-3% feet proteins), 230 kD (9-13%), 160 kD (1.8-2.5%), 64 kD (2.0-4.7%), 24 kD (1.4-3.0%), 200 kD (1.0-1.0% myosine heavy chain?), 90 kD (0.6-1.9% phosphorylase?). Components at 200 and 90 kD are not visible in FSRs samples. These protein components can be removed from the FSR-C by washing with 0.6 M KCl (FSR-Cw, Table 1).

Table 1. Relative molecular weight and quantitative distribution of proteins in different FSR samples. The distribution of the components in percentage were determined after SDS PAGE by densitometry

Sample	molecular mass of proteins, kD										
	340-370	230	200	160	105	90	64	55	45	32	24
	relative distribution in percent										
FSR-S	2.8	13.6	-	2.0	64.7	-	2.4	3.8	6.6	2.5	1.6
FSR-N	1.9	9.8	1.0	1.8	68.0	0.6	2.0	3.8	6.6	2.1	2.4
FSR-C	1.9	10.7	1.2	3.0	64.0	1.7	2.9	4.8	6.1	2.3	1.4
$FSR\text{-}C_w$	2.5	9.0	-	3.5	64.2	-	4.7	4.9	5.6	2.5	3.1

Metals are frequently thought to have influence on the secondary, tertiary and quaternary structures of proteins and hence on their function and conformation. Therefore we determined the calcium, magnesium and zinc concentration in our FSR samples (Table 2). The magnesium and calcium concentrations were 10-34 nmol/mg protein and 78-136 nmol/mg protein, respectively. Calcium:magnesium ratio of samples washed with 0.6 M KCl (FSR-S, FSR-N, FSR-Cw) was slightly higher than that in FSR-C. The Zn concentration was 1.79-5.35 nmol/mg protein which corresponds to 0.25-0.83 nmol Zn per nmol ATPase content. Table 2 also shows that the Zn concentration is higher in samples washed with KCl solution.

Table 2. Calcium, magnesium and zinc content of FSR samples

Sample	Calcium	Magnesium	Zinc
	nmol/mg protein		
FSR-S	136.0	28.2	3.37
FSR-N	92.3	19.2	2.56
FSR-C	80.0	34.2	1.79
$FSR-C_w$	77.9	10.2	5.35

Mg-activated PNPPase activities of the various FSR samples were similar but their Mg-activated ATPase activities were considerably different (Table 3). There is a correlation between the Ca + Mg-activated PNPPase and Ca + Mg-activated ATPase activities because the specific activities decrease in the FSR-C>FSR-S>FSR-N order.

Table 3 also shows that these specific activities are lower in those FSR-S and FSR-N samples, which were washed with 0.6 M KCl than in FSR-C. However, ACEase activities change in the reverse manner. Ca + Mg-activated ATPase activity of FSR-C, when washed with 0.6 M KCl, was similar to those of FSR-S and FSR-N. ACEase specific activity of FSR-C was only slightly in-

fluenced by washing the sample with KCl (FSR-Cw). SOD specific activities of FSR-N and FSR-S were higher than that of FSR-C which was not washed with KCl (Table 3). Table 2 reveals also that the Zn concentration in FSR-S and FSR-N is higher than that in FSR-C. The correlation between the Zn content and SOD specific activity could correspond to the Cu, Zn-containing metalloprotein nature of SOD.

Table 3. p-Nitrophenylphosphatase, ATPase, acetylcholinesterase and superoxide dismutase activities in the various FSR samples

Enzyme	FSR-S	FSR-N	FSR-C	FSR-C_w
	specific activity*			
Mg^{2+} activated PNPPase	4.57	4.35	4.75	n·d
Mg^{2+} activated ATPase	1350.00	570.00	970.00	550.00
Ca^{2+} + Mg^{2+} activated PNPPase	9.63	9.18	15.50	n·d
Ca^{2+} + Mg^{2+} activated ATPase	3450.00	3000.00	4050.00	3220.00
ACEase	13.00	7.10	2.80	3.00
SOD**	5.90	5.67	5.20	n·d

* specific activity: nmol product/mg protein per minute;
** U/mg protein;
n·d = not determined

Determination of SH-group reactivity is a sensible probe for the conformation of several soluble proteins (14). In the FSR one can found 6-8 mol SH-groups with DTNB without previous detergent treatment (16). The SH-groups are divided into two groups, according to their function and kinetic reactivity with DTNB i.e. there are fast and slow ones (2, 3, 16, 28, 42).

Table 4. SH-content of different FSR samples in Ca + EGTA as well as in EGTA containing incubating solutions, nmol SH per mg FSR protein

Sample	Time in minute															
	0.2	2.0	5.0	10	20	30	40	60	0.2	2.0	5.0	10	20	30	40	60
	in Ca + EGTA								in EGTA							
FSR-S	1.5	6.5	11.5	20.0	30.5	47.5	56.5	70.4	3.0	12.0	20.0	32.0	46.5	56.5	67.5	82.0
FSR-N	4.2	8.9	12.0	18.0	31.0	41.5	52.0	68.0	6.6	18.5	25.0	35.0	49.0	58.0	66.0	77.0
FSR-C	7.4	14.5	21.5	28.5	40.0	48.5	57.0	71.0	2.7	9.0	17.8	26.0	37.0	45.0	51.0	62.7
FSR-C_w	5.9	9.5	12.6	17.4	26.6	32.5	41.0	52.5	6.1	23.8	27.5	33.3	40.7	47.3	52.5	61.5

Table 5. Time constants of fast and slow SH-groups in different FSR samples reacting with DTNB

Sample	in Ca + EGTA				in EGTA			
	containing incubating solution							
	time constant		amount of SH-s		time constant		amount of SH-s	
	minute		nmol SH/mg protein		minute		nmol SH/mg protein	
	fast	slow	fast	slow	fast	slow	fast	slow
FSR-S	0.70	30.0	6.3	64.1	0.40	20.0	10.6	71.4
FSR-N	0.42	26.0	6.8	61.2	0.40	17.4	13.9	63.1
FSR-C	0.35	21.3	11.4	59.6	0.66	18.0	8.1	54.6
FSR-C_w	0.40	25.8	7.3	46.2	0.28	18.8	20.9	40.6

Semilogarithmic depiction of the timescale of this reaction has two linear parts from which the time constant and SH-concentration can be determined (16). We have found that the method of FSR isolation and the medium of SH-determination reactions strongly affect the reactivity of the SH-groups (Table 4). Namely, in the case of FSR-S, FSR-N, FSR-Cw, which were washed with KCl in a medium containing EGTA, more SH-groups could be determined with a 40-45% higher kinetic reactivity (smaller time constant) than in a medium containing Ca + EGTA (Table 5). In the case of FSR-C which was not washed, the reverse situation can be observed (Table 4-5).

DISCUSSION

Our results on the distribution of proteins from the different FSR samples are similar to literature data (25, 24, 32). There was no significant difference found in the amount of 100 kD Ca-pump protein when the FSR samples independent of washing the samples with 0.6 M KCl (Fig. 1, Table 1).

Zn content of FSR-S, FSR-N and FSR-Cw was usually twice as high as that of FSR-C which was not washed with KCl solution. Calcium:magnesium ratios were higher in the former case, as well. Recently, Papp et al. (30) reported metal-ion concentration values of FSR-N isolated, according to Nakamura et al. (29). Their measurements show 2-3 times less calcium, zinc and magnesium than that found in our results. Nevertheless, their and our calcium:magnesium, calcium:zinc and magnesium:zinc ratios are very similar. Earlier we have examined (6) calcium, magnesium and zinc concentratios of fish muscle FSR samples, washed with 0.6 M KCl. Using atomabsorption spectroscopy we found 39.2 nmol Ca, 6.8 nmol Mg and 5.2 Zn for 1 mg of FSR protein. These are considerably different from the metal ion concentrations of rabbit muscle FSR (Table 2). This finding reveals that the metal-composition of FSR depends not only on the isolation method but on the species, as well.

Ca + Mg-activated PNPPase, ATPase and ACEase specific activities of our FSR samples (Table 3), which were washed with 0.6 M KCl when isolated, e.g. FSR-N, are similar to those

in literature (32, 46, 47). It is known that FSR has ACEase activity as well (15, 19, 35, 37, 45). We have found (21, 22, 23, 38) that there is a correlation between changes in functional properties i.e. Ca-influx, Ca + Mg-dependent ATPase activity, of the FSR and changes in ACEase activity. Table 3 shows that Ca + Mg-dependent ATPase activities of KCl washed FSR-S and FSR-N are lower, whereas their ACEase activity is higher than that of FSR-C. The differences are well represented by the Ca + Mg-dependent ATPase:ACEase ratios. These are 265, 422, 1446 and 1073 for FSR-S, FSR-N, FSR-C and FSR-Cw, respectively. Values for FSR-N and FSR-S are very similar, 451 and 234, to those determined from the results of Sarzala et al. (32) and Vidal et al. (47). They have isolated their FSR samples either in a KCl containing medium or washed them with 0.6 M KCl during isolation. Considering our earlier observations (21-23, 38) we presume that there are correlations between the ratios of ATPase:ACEase activities and the degree of protein-protein and protein-lipid interactions in the SR membrane, due to the randomness and conformation of the membrane.

It was already mentioned that the reactivity of SH-groups is a sensible probe of the conformation of the proteins. It was reported that the SH-reactivity of FSR is increased in the presence of EGTA (3, 30). This was corroborated by our data (Table 4-5) arising from DTNB titration of the SH-groups of the FSR-S, FSR-N, FSR-Cw samples. However, in the case of FSR-C, without washing with 0.6 M KCl, it was the Ca + EGTA-containing medium where the reactivity of both fast and slow SH-groups were increased. The difference in the SH-reactivity is probably due to the different conformations of the two samples. For example, Papp et al. (30) found that the reactivity of SH-groups increased by decreasing Zn concentration and vice versa. This interaction appeared to be nonlinear. We have also observed a similar correlation when the effect between Ca, Mg and Zn concentrations and reactivity of SH-groups were examined. The Zn concentration of FSR-C is 1.79 nmol/mg protein. When the amount of Zn is 40-45% higher (FSR-N: 2.56, FSR-S: 3.37, FSR-Cw: 5.35 nmol Zn/mg protein) the number of SH-groups that can be meas-

ured increases in the EGTA containing medium as compared to the medium containing both Ca and EGTA (Table 2, 4, 5). This finding is supported by the observed correlation between the Zn content and SH-reactivity of light and heavy microsomes isolated by density gradient centrifugation from the FSR-C (40). Namely, the SH-reactivity of light microsome of low zinc concentration (2.0 nmol Zn/mg protein) is lower in a medium containing only EGTA. In contrast, the SH-reactivity of the heavy microsome containing more Zn (9.35 nmol Zn/mg protein) is higher in a similar medium.

When the samples were washed with 0.6 M KCl (see FSR-C and FSR-Cw in Table 1) the 200 kD (1.2%, myosine heavy chain) and 90 kD (1.7%, phosphorylase) components were removed. Since the amount of these extracted proteins is low, 2,9%, probably they do not have significant influence on the SH-reactivity of FSR-C (see FSR-N in Table 1). When the FSR is washed with 0.6 M KCl the nucleotides (ATP, ADP) are removed, as well (3). However, the enzyme-bound nucleotides do affect the reaction of DTNB and FSR-ATPase, e.g. in FSR-C, because they are bound to the binding-sites close to the reactive SH-groups or they can induce conformation changes in the FSR-ATPase.

In conclusion, the amount of Zn, washing with 0.6 M KCl and fractionation on sucrose density gradient alter the conformation of FSR which is indicated by the changes in the SH-reactivity of SH-groups.

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NEW APPROACH OF THREE-DIMENSIONAL CRYSTALLIZATION OF THE Ca^{2+}-ATPase OF SARCOPLASMIC RETICULUM

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SUMMARY

Selective extraction procedure was applied for obtaining different proteins from sarcoplasmic reticulum vesicles following the main steps of previous observations (21, 23). High concentration of a nonionic detergent, such as polyoxyethylene-10-lauryl ether ($C_{12}E_{10}$) prevented the formation of Ca^{2+}-ATPase crystals. It has been observed that only the 300 kDa protein could induce crystallization from among proteins being undissolved from of membrane.

A modification of MacLennan's procedure - applied for ATPase precipitation from deoxycholate solubilized sarcoplasmic reticulum - has been described and an ammonium acetate precipitated Ca^{2+}-ATPase was used in the experiments for increase of Ca^{2+}-ATPase concentration in the crystallization process. The repetead supplementation of purified and $C_{12}E_{10}$ solubilized Ca^{2+}-ATPase with ammonium acetate precipitated Ca^{2+}-ATPase made possible a formation of larger and larger crystals with different periodicity.

Abbreviations: BRIJ-36T, polyoxyethylene-10-lauryl-ether; $C_{12}E_8$, octaethylene glycol dodecyl ether; $C_{12}E_{10}$, polyoxyethylene-10-lauryl ether; Ca^{2+}-ATPase, Ca^{2+}-ion activated ATPase; CaBP, high-affinity Ca^{2+}-binding protein; CaS, calsequestin; DOC, deoxycholate; DTT, dithiotreitol; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N'N'-tetraacetic acid; HMW, high molecular weight; LMW, low molecular weight; MOPS, 3-(N-morpholino) propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SR, sarcoplasmic reticulum; SRV, sarcoplasmic reticulum vesicles.

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INTRODUCTION

Studies on covalent crosslinking of Ca^{2+}-ATPase by a variety of crosslinking reagents both in native SR and in detergent solubilized preparations revealed the characteristics and conditions of self-association or crystallization i.e. the formation of three dimensional arrays of enzyme molecules (1-5, 23). Most studies suggested that dimers form part of crosslinked products of different size, among them very large polymers (1-2, 6, 7-10). Most of the reagents produced a variety of ATPase polymers with no clear indication of a preferred size of oligomers.

Detergents such as Triton X-100 (3, 8), BRIJ-36T, $C_{12}E_8$ (2, 23) or DOC (9) could generally reduce the extent of crosslinking. Particularly at higher detergent concentrations almost a complete conversion of the Ca^{2+}-ATPase into monomeric form occured, even at relatively high protein concentrations (11, 2-3, 8-9). Besides, depending on the type of detergent, both the amount of phospholipid associated with the enzyme and the specific activity of Ca^{2+}-ATPase was diminished as a function of time.

In previous studies on crystallization of Ca^{2+}-ATPase in the presence of detergent we observed that the preservation of ATPase activity and the formation of three dimensional arrays of Ca^{2+}-ATPase molecules was the best at pH 6.0, and it required the presence of 20% glycerol and 20 mM $CaCl_2$ in the medium (12, 4, 5). Furthermore our data suggested a relationship between the function of ATPase aggregates and the long-term stability of the enzyme (4). These observations on crystallization of Ca^{2+}-ATPase emphasized the requirement of a homogeneous population being in either E_1 or E_2 conformation of enzyme molecules for the occurence of crystals even in two dimensions. The three dimensional crystallization, however, would need further alteration of the conditions, among them the optimalization of protein/lipid/detergent ratios as well as the increase of protein concentration in the medium to as high as possible. Furthermore, the crystallization itself requires an additional supply of Ca^{2+}-ATPase in incubation mix-

ture. A further question is whether the presence of insoluble membrane components in medium could promote the crystallization. This paper will describe some of the details.

MATERIALS AND METHODS

All reagents and standards (HMW, LMW) used in PAGE were obtained from Bio-Rad, Pharmacia Fine Chemicals, Serva and Shandon. All other chemicals were at least reagent grade and were purchased from Eastman, Merck, Reanal and Sigma Chemical Companies.

FSR was prepared from rabbit muscle practically in a way as described by Nakamura et al. (13). The preparations were kept frozen in liquid nitrogen and stored before use in a medium containing 0.3 M sucrose, 10 mM Tris-maleate buffer, pH 7.0, at a protein concentration of 20-30 mg/ml in polyethylene containers at -70°C. Immediatelly before the experiments the microsomes were thawed and to remove sucrose, SR vesicles were diluted 10-fold with standard medium containing 0.1 M KCl, 10 mM K-Mops, pH 6.0, 3 mM $MgCl_2$, 5 mM DTT, 3 mM NaN_3, 25 IU/ml Trasylol, 2 µg/ml 2,6-di-tert-butyl-p-cresol and centrifuged.

For extraction of Ca^{2+}-ATPase and CaBP with $C_{12}E_{10}$ (21) the pellet was resuspended (10 mg protein/ml) in a standard medium containing 20 mM $CaCl_2$ and 10% glycerol and then extracted at 2°C with $C_{12}E_{10}$ (10 mg/ml) for 30 min. After centrifugation at 2°C, 130 000 g for 30 min the pellets were resuspended in the same media and reextracted with detergent using $C_{12}E_{10}$ at a ratio of 5 mg detergent/mg protein and a final concentration of 50 mg/ml.

The insoluble material - containing CaS and the 300 kDa protein - was removed by centrifugation (21), washed twice with a solution of 50 mM imidazole, pH 8.0 and reextracted with the same solution containing 10 mM EGTA. After centrifugation the supernatant was dialyzed against a solution of 10 mM imidazole, pH 7.0 and the precipitated CaS was collected and suspended in the same solution. The final pellet obtained by centrifugation was extracted with a solution of 20 mM imidazole, pH 8.0, containing 1.0 M KCl and 20 mM $CaCl_2$ for solubilizing the 300 kDa protein. After removal of insoluble material with centrifugation the supernatant was dialyzed against a solution of 10 mM imidazole, pH 7.0, containing 20 mM $CaCl_2$. The 300 kDa protein was first precipitated, then collected and finally suspended in 10 mM imidazole solution, pH 7.0.

For the analysis of protein composition by SDS-PAGE the samples were dissolved in a solution of 5% SDS, 10 mM Tris-HCl buffer, pH 8.0, 1% 2-mercaptoethanol and 10% glycerol, at a protein concentration of 1-2 mg/ml. Aliquots of about 50 µl were applied for electrophoresis on 6-12% gradient gels, according to Laemmli (14).

The Ca^{2+}-activated ATPase activity was measured either by a coupled enzyme system of pyruvate kinase and lactate dehydro-

genase (15, 16) or by the analysis of inorganic phosphate liberation from ATP according to Fiske-Subbarow (17).

Phospholipids were extracted with chloroform-methanol mixture (18) and analyzed by thin-layer chromatography, as described by Sarzala and Michalak (19). The phospholipid phosphorus was determined by measuring the inorganic phosphorus released by treatment with perchloric acid at 200°C, according to Bartlett (20).

The crystallization of Ca^{2+}-ATPase was carried out at 2°C in standard crystallization medium, which is similar to standard medium containing 10-20% glycerol and 20 mM $CaCl_2$ as described by Dux et al. (12) and the structure of Ca^{2+}-ATPase microcrystals was observed after negative staining with 1% uranyl acetate (4) with a JEOL 100B electronmicroscope at 80 kV accelerating voltage.

Protein concentrations were estimated, according to Lowry (22) using bovine serum albumin as reference.

RESULTS AND DISCUSSION

In earlier studies it was shown that the presence of 10-20% glycerol and 20 mM Ca^{2+} in the extraction solution renders possible a relatively selective and successive separation of proteins of different physicochemical properties from SRV by some nonionic detergents (21). At 1 mg detergent/1 mg protein ratio practically only the CaBP is dissolved from membrane, all other proteins remained in the residue. After removing CaBP by centrifugation the pellet was suspended in the same solution but at a higher detergent concentration (4-5 mg detergent/mg protein). Under these conditions only the Ca^{2+}-ATPase monomers were dissolved while dimers and oligomers of Ca^{2+}-ATPase, i.e. feet proteins (300 kDa protein) the CaS and all stroma proteins remained in pellet on centrifugation. The second step of the preparative procedure resulted in a highly purified Ca^{2+}-ATPase enzyme preparation that preserved activity for a long time.

If in the first step a relatively high detergent concentration (4-5 mg detergent/mg protein) was used, Ca^{2+}-ATPase was solubilized together with the CaBP. In the further steps the high molecular weight "feet" protein could be extracted from the pellet with a solution that also contained 1 M KCl. Finally for dissolving the CaS a medium containing EGTA instead of Ca^{2+}

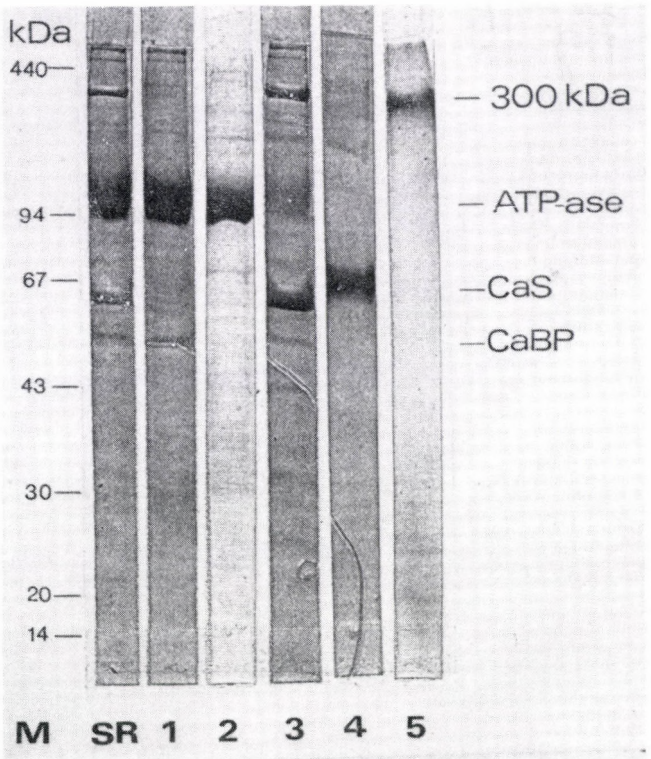

Fig. 1. SDS-polyacrylamide gel electrophoretic patterns of SR membrane proteins after selective extraction in the presence of nonionic detergent ($C_{12}E_{10}$). Lanes: M, molecular weight marker; SR, as control;
1. Ca^{2+}-ATPase + CaBP after extraction with 5 mg $C_{12}E_{10}$/mg protein; 2. Ca^{2+}-ATPase after successive extraction with 1 mg $C_{12}E_{10}$ and 5 mg $C_{12}E_{10}$/mg protein; 3. CaS and 300 kDa protein; 4. CaS; 5. 300 kDa protein

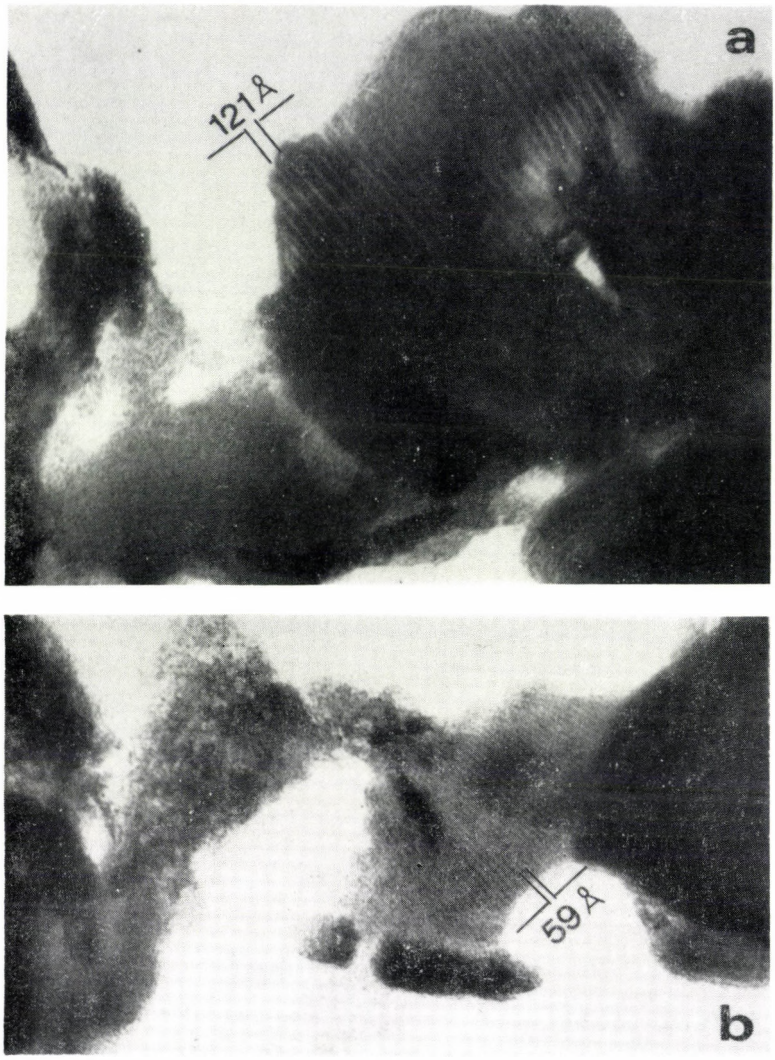

Fig. 2. Ca^{2+}-ATPase microcrystals obtained in reconstructed system of membrane components in the presence of $C_{12}E_{10}$. Preparation of Ca^{2+}-ATPase and other membrane proteins was carried out essentially as described in Materials and Methods. After selective extraction a system was made of membrane components (Ca^{2+}-ATPase + CaS + 300 kDa protein) in standard crystallization medium with $C_{12}E_{10}$ and incubated for at least 7 weeks.

Part a: microcrystals of large periodicity after incubation for 7 weeks. (crystal lattice spacing: 121 Å; magnification, × 140,250).

Part b: "final" crystal form of smaller periodicity after incubation for 10–15 weeks. (crystal lattice spacing, 59Å; magnification, × 140,250)

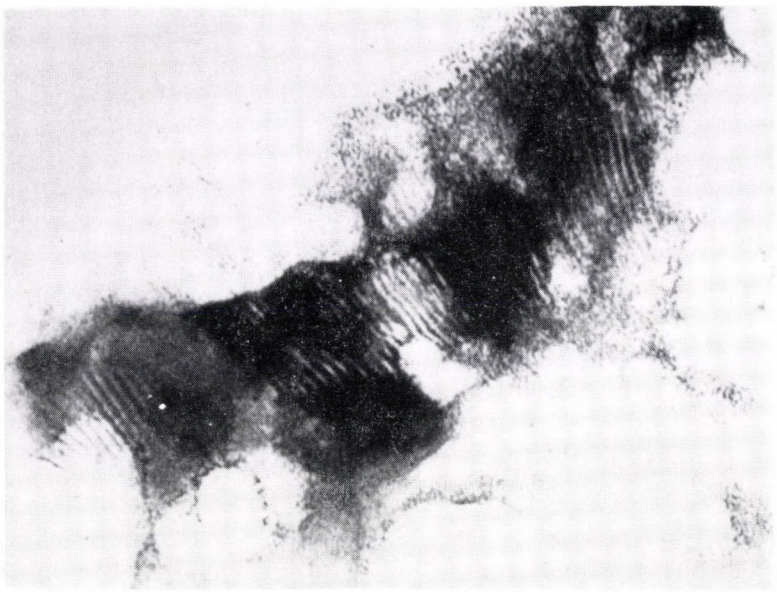

Fig. 3. Ca^{2+}-ATPase microcrystals of reconstructed system of membrane components (Ca^{2+}-ATPase + CaS) in crystallization medium with $C_{12}E_{10}$. Picture was taken after two weeks of "replace" of CaS. Magnification, × 102,300

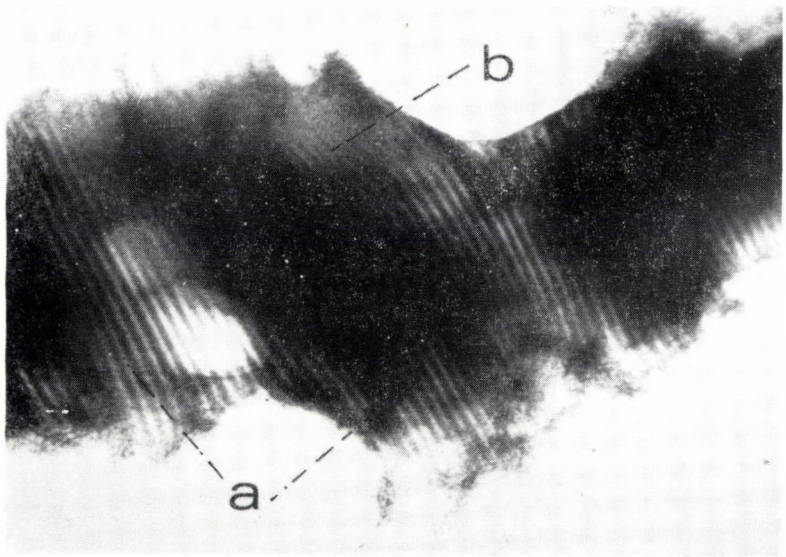

Fig. 4. Microcrystals of Ca^{2+}-ATPase + 300 kDa "feet" protein reconstructed system after 7–10 days of "replace" of 300 kDa protein. a. microcrystals of large periodicity (crystal lattice spacing: 149Å, magnification, × 102,300. b. small periodicity form (crystal lattice spacing: about 65 Å)

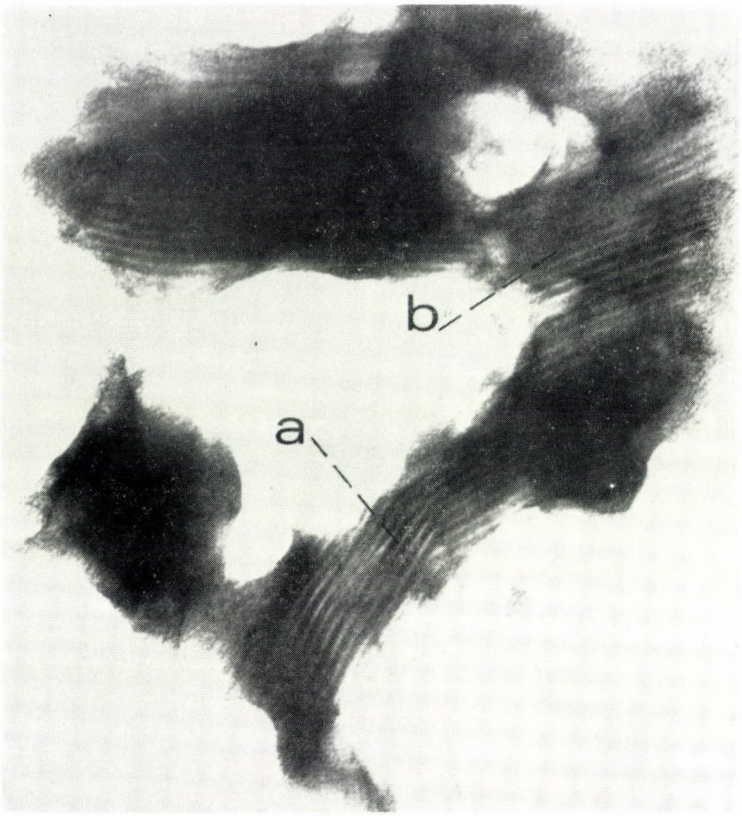

Fig. 5. Microcrystals of Ca^{2+}-ATPase + ammonium acetate precipitated Ca^{2+}-ATPase system after 3–4 days of incubation (magnification, × 102,300)
a. large periodicity form, crystal lattice spacing: 152 Å
b. small periodicity form, crystal lattice spacing: 60 Å

was applied at pH 8.0. These last two steps were interchangeable. In the final pellet remained the insoluble stroma and the high molecular weight polimers of ATPase. SDS-PAGE was performed of some samples taken in course of each procedure as shown in Figure 1.

Proteins isolated by this way from SR were used in the standard crystallization medium to learn how their presence can help for the crystallization of Ca^{2+}-ATPase.

In experiments on crystallization of Ca^{2+}-ATPase in SR (suspended in a standard crystallization medium) a partial solubilization of membrane has been required to induce crystallization process itself. The treatment of membrane by detergent reduced the free phospholipid content and simultaneously increased the capability of ATPase molecules for lateral movement and their local concentration for aggregation. As a consequence, crystallization has occurred. But the crystallization index decreased significantly when the detergent protein ratio(4-5 mg $C_{12}E_{10}$/mg protein) was raised (4, 5, 12). Even the crystals formed previously could be broken and they may disappear on the increase of detergent/protein ratio. The use of a high detergent concentration, however, is essential for solubilizing totally the ATPase from membrane.

Crystallization of Ca^{2+}-ATPase cannot be observed in the supernatant obtained by centrifugation of SR suspension after solubilization at high detergent/protein ratio, independent of CaBP had been removed at low detergent/protein ratio, previously.

In series of experiments 1.) CaS + 300 kDa protein; 2.) CaS and 3.) 300 kDa protein in different amounts (about 2.5, 5, 10 or 20% of total amount of protein) were measured in plastic tubes used for crystallization, then detergent solubilized, purified ATPase (obtained by successive extraction) was pipetted into tubes in quantities to reach the required ratios of various proteins. These results are presented in Figure 2, 3 and 4.

Only negligible crystallization was observed when CaS even at a 20 or 30% portion was present (Fig. 3). CaS and

300 kDa protein (10-10%) as well as 300 kDa protein alone at about 20% could induce a very well expressed crystallization of Ca^{2+}-ATPase (Fig. 2 and 4). These results may indicate that only the 300 kDa protein is an efficient inducer for the crystallization process.

First, according to MacLennan's studies (24), the ammonium acetate precipitation was tried to be applied without any modification except using $C_{12}E_{10}$, a nonionic detergent, instead of DOC for precipitating ATPase, but no precipitate was formed even at much higher concentration of ammonium acetate. $C_{12}E_{10}$ can prevent precipitation of Ca^{2+}-ATPase when applied at a high concentration enough to dissolve totally enzyme from membrane. Considering these results, the reduction of detergent concentration and an increase of that of ammonium acetate seems to be required in otherwise the same crystallization medium to precipitate Ca^{2+}-ATPase.

A few ml of Ca^{2+}-ATPase (obtained by successive extraction at a detergent/protein ratio of 5, and protein concentration of 5-10 mg/ml) were poured into a dialysing bag and was dialysed overnight against 100 volumes of crystallization medium saturated to 50% with ammonium acetate. The precipitated Ca^{2+}-ATPase was collected by centrifugation (130 000 x g, $2^{o}C$). The sediment was slightly yellow and almost transparent indicating that a part of phospholipids remained associated with the enzyme. Under such conditions the total and specific activity of Ca^{2+}-ATPase remained constant.

In the crystallization the different amounts of undissolved, ammonium acetate precipitated Ca^{2+}-ATPase sediment were put into tubes and 0.5-1.0 ml of Ca^{2+}-ATPase solution (obtained by successive extraction) was added to it. Tubes containing both dissolved and undissolved Ca^{2+}-ATPase at different ratios were stored for weeks at $2^{o}C$, eventually taking samples for electronmicroscopic control (Fig. 5).

A part of the precipitate was dissolved immediately after pipetting Ca^{2+}-ATPase solution into it indicating that the $C_{12}E_{10}$ remained in excess in the solution. This procedure seemed to be convenient to obtain true three-dimensional crystals of purified Ca^{2+}-ATPase of larger size.

Electronmicroscopic studies at first crystals of larger (crystal lattice spacing: 120-160 Å) then smaller periodicity (crystal lattice spacing: 55-65 Å) were detected during crystallization. This can possible be connected with the changes in the extent of hydratation of molecules.

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BOOK REVIEWS

IRRITATION; Chemical Senses Series, Volume 2. Edited by: Barry S. Green, J. Russel Mason and Morely R. Kare. Marcel Dekker, Inc. (270 Madison Ave, New York, N.Y. 10016) 1990. 384 pages, USD 119.50 (ISBN 0-8247-8323-9)

The Monell Chemical Senses Center celebrated its twentieth anniversary in 1988 by organizing four international meetings on all aspects of the chemosensory research. This volume is based on the second symposium which was devoted to discussion of the chemical irritation in the nose and mouth. The book which contains not only the presentations but also the discussions, is recommended for anatomists, physiologists, psychophysicists, biochemists, biologists, behavioral and food scientists and graduate students in these disciplines.

The chapters cover a wide variety of topics including anatomy, pharmacology of nerve fibers sensitive to nasal irritants or psychology of chemoreception. Experimental data are presented about the affector or effector functions of peptidergic innervation, the physiology of nasal trigeminal chemoreception and the interactions between trigeminal afferents and olfactory receptor cells.

Other presentations characterize the brain response to chemical stimulation of trigeminal nerves, compare the effects of oral irritants, survey the significance of individual variation in the perception of irritation. A comprehensive chapter describes the results of electrophysiological and morphological experiments related to the effects of capsaicin and to the properties of the capsaicin-sensitive sensory neurons.

A separate chapter is dealing with the botany, cultural history and pharmacology of chili pepper which is one of the most widely consumed substances in the world.

Some properties of the nasal sensory receptors, the mechanism of irritants action were discussed and practical applications were given in the symposium to protect workers and consumers from the toxic effects of the airborne chemicals.

The last chapter is the General Discussion which makes it apparent that the symposium producing more questions than answers can be a stimulus and guide for future research about the chemical sensitivity of the oral and nasal mucosa.

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Department of Physiology
Univ. Med. School of Debrecen

BIOSENSOR TECHNOLOGY, FUNDAMENTALS AND APPLICATIONS: Edited by Richard P. Buck, William E. Hatfield, Mirtha Umana and Edmond F. Bowden. Marcel Dekker, Inc. (270 Madison Ave, New York, N.Y. 10016), 1990, 408 pages, USD 119.50, ISBN 0-8247-8414-6

This book contains the proceedings of the International Symposium on Biosensors, which was held on the campus of the University of North Carolina at Chapel Hill on September 7-9, 1989. The program covered the recent advances of the field, emphasized the interdisciplinary contribution by physicists, electrical engineers, analytical chemists, biochemists, immunologists, etc. in the development of biosensor technology. Fundamental issues of sensitivity and selectivity were discussed wherever possible.

Following an introduction to the field of biosensors the first section consists of four chapters devoted to microelectrodes and microelectronic devices. The solid state potentiometric sensors are the product of integration of solid state device physics and electrochemistry. These sensors can replace the traditional ion-selective and enzyme electrodes and

using the chemical modulation of electron work function are convenient for potentiometric sensing in gases and dielectric fluids. For in vivo measurements the investigators achieved a small size and succesfully determined, e.g. the dopamine release in the brain.

The ten chapters of the next section are dealing with the modified electrodes, amperometric and potentiometric sensors. One of the most interesting approaches is the mimicking of biological transmembrane signalling processes. Novel sensing membranes for potentiometric and voltametric detection of organic substances have been developed by the use of lipophilic derivatives of macrocyclic polyamine and related compounds as membraneous receptor molecules. The combination of redox enzyme catalysts and amperometric electrochemistry resulted in the development of amperometric enzyme electrodes, using dehydrogenases and flavoproteins. The neuroreceptor-based biosensors consists of receptor proteins with highly selective binding affinities for recognition and non-biochemical materials to get discernable readout signals. The enzyme-analyte conjugates can be signal generators for amperometric immunosensors. A competitive solid-phase immunoassay is considered with the immunoglobulin immobilized on a flat surface, preferably directly on that of the electrode or in its close vicinity. Several chapters focus the polypyrrole glucose biosensors which renders possible an accurate and inexpensive method of glucose determination in biological fluids.

The final section of fourteen chapters presents experimental data about the optical and acoustic wave-based sensors. The homogenous immunoassay can be performed by optical fiber electrodes. Multi-channel immunosensors are developed for remote and continous monitoring of biologically active substances. The atomic force microscope can be operated in an aqueous environment and is capable of providing real time images of protein adsorption with a resolution sufficient to see individual molecules. Fiber optic-based biosensors utilizing thermal sensors, polymer films and immobilized enzyme systems are described in details.

Laszlo Kovacs

GUIDE TO FLOW CYTOMETRY METHODS W. McLean Grogan and James M. Collins, Marcel Dekker Inc. (170. Medison Ave, New York, N.Y. 10016), 1990, 228 pages, USD 119.5, ISBN 0-8247-8330-1

This volume cannot be regarded as a handbook of flow cytometry but instead it is really a guide to the wide applications of this technique. It is written for researchers that are more interested in the kinds of analyses that can be performed by flow cytometers, a short and concise theory behind particular types of measurements, in practical advices and recipes, than in having an exhaustive review of the technique itself and the achievements done by flow cytometry. Each chapter contains a list of references (original papers) and some of them offer also suggested readings for those having an interest to learn more about the topics.

The first chapter is an introduction giving a brief description of flow cytometers. A really short account is also given on fluorescence, optical filters, data collection and safety rules in a flow cytometry laboratory.

Preparation of single cell suspension is described in the second chapter including detailed protocols for solid tissue dispersal, Ficoll gradient purification of peripheral blood lymphocytes, preparation of bone narrow cells, etc.

Different methods for DNA staining and the applications of DNA analysis is the subject of the third chapter which is the most extensive one of the book with more than 150 references. The chapter also includes eleven staining protocol and the discussion of the prognostic value of DNA analysis for certain types of neoplasm.

Viability assays are discussed in chapter four, and the information content of light scattering signal is outlined in chapter five with a review oftheory of light scattering process. Chapter six deals with immunofluorescence including direct and indirect staining. Chapter seven is intended to give the reader sufficient background and a practical guide to cell sorting.

Mitochondrial stains, measurement of intracellular pH, intracellular Ca, intracellular glutation, membrane fluidity and membrane potential are covered by the short chapters No. 8-12 and 14, respectively.

Chapter 13 deals with the determination of the number of cell surface receptors, and the binding of antibodies to cellular antigens as important tools in cellular identification and classification. Chapter 15 is a compilation of miscellaneous applications

not discussed in detail.

In the appendix fluorescence parameters of the most frequently used dyes are given.

Guide to Flow Cytometry Methods is primarily written for clinical researchers. The background and procedures provided should also constitute a valuable resource to clinical flow cytometry laboratories.

Lajos Trón

GENERAL INFORMATIONS

1st WORLD CONGRESS OF CELLULAR AND MOLECULAR BIOLOGY

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FAST COMPONENTS OF THE ABSORPTION CHANGES OF BACTERIORHODOPSIN AT 275 NM and 296 NM

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SUMMARY

A very fast component (life time 0.2 μs) was found in the flash-induced absorption changes of bacteriorhodopsin (bR) at 275 nm and 296 nm. This result was obtained by measuring the absorption changes at well defined delay times after the exciting laser flash (590 nm, 20 ns pulse duraiton). For this purpose a second laser flash was used as the monitoring beam. The very fast absorption changes of bR in the UV range are due to the rapid perturbation of the opsin moiety near the chromophore, as a result of the all-trans to 13-cis isomerization of the retinal taking place on the same time scale.

INTRODUCTION

Bacteriorhodopsin (bR) is a membrane chromoprotein found in the cell membrane of Halobacterium halobium, where it functions as a light-driven protein pump (Stoeckenius, Bogomolni, 1982).

Upon absorption of a photon a photocycle is initiated, which proceeds through a set of spectroscopically distinct intermediates (Lozier et al., 1975). At least four intermediates exist in the time range from 0 to 5 μs (Midler, Kigler, 1988). During these fast transitions the retinal chromophore undergoes isomerization from its all-trans to the 13-cis form.

In the UV range the absorption changes of the bR after photo-excitation have a minimum at 275 nm and a maximum at 296 nm (Czege et al., 1982) which have been tentatively assigned to the perturbation of the protonation states of the tyrosine and tryptophane amino acid residues, respectively (Hess, Kuschmitz, 1979; Bogomolni et al., 1978;

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Hanamoto et al., 1984; Dupuis et al., 1985). In the μs range (>1 μs), at 296 nm and 275 nm, two kinetic components have been found at neutral pH and room temperature (24+1 ^{o}C). The faster component has been assigned to the chromophore isomerization, the slower one to the perturbation of aromatic amino acids (Dupuis et al., 1985; Jang et al., 1990). At 1.5^{o}C a very fast component (<1 μs, not time resolved) has also been proposed, and attributed to a charge shift in the environment of the aromatic amino acid residues (Kuschmitz, Hess, 1982). The use of continuous UV light in all these experiments may, however, lead to degradation of the sample, and at low temperatures side effects may interfere with the results. Therefore a more precise measurement of the absorption changes at 275 nm and 296 nm is desirable.

In this paper an experiment based on a different approach for measuring very fast absorption changes of bR in the UV after photoexcitation is described.

MATERIALS AND METHODS

Bacteriorhodopsin, in the form of pm sheets, was isolated from Halobacterium halobium, strain ET-1001 according to a standard procedure (Oesterhelt, Stoeckenius, 1974). The pm-s were immobilized in polyacrylamide gel as described previously (Der et al., 1985). The gels were further equilibrated in an universal buffer solution (containing citric acid, monopotassium hydrogensulfate, borate and diethylbarbiturate) at pH 7. The absorbance of the sample in a quartz cuvette of 2 mm thickness was 1.2 at 568 nm. All measurements were carried out at room temperature (24^{o}C).

The flash-photolysis setup used for measuring the fast absorption changes of the bR at 275 nm and 296 nm is presented in Fig. 1. The photocycle was initiated by flashes from a dye laser pumped by an excimer laser (Lambda Physik, FRG, EMG 101 MSG,). The dye Rhodamine 6G (Lambda Physik, FRG, emission maximum 590 nm) was used as the active medium. The flux density of the exciting laser flash energy was estimated to be 8 mJ cm^{-2} (2.4x10^{16} photons cm^{-2}) at the sample. This photonflux is dense enough for maximal excitation of the bR molecules. For the monitoring beam, a laser pulse from a second dye laser pumped by another excimer laser (JATE, Hungary) was used. The monitoring pulse was focused on a KDP

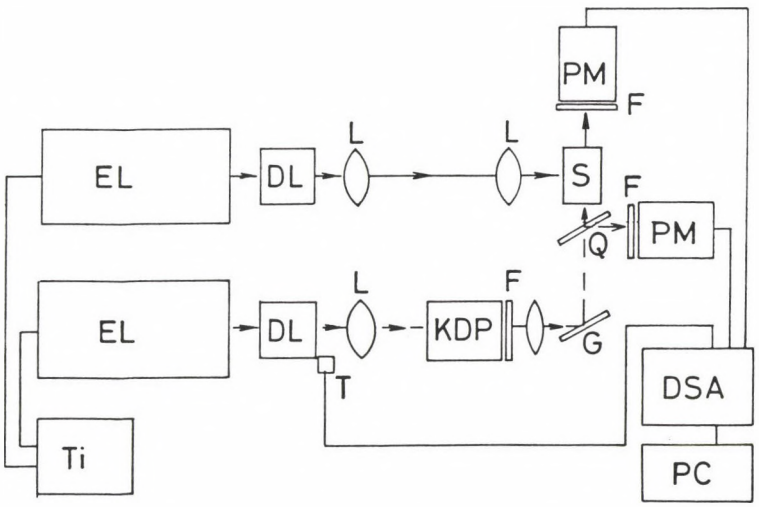

Fig. 1. Flash-photolysis setup for measuring the fast absorption changes of the bR. EL - excimer laser, DL - dye laser, L - lens, KDP - potassium dihydrogenphosphat crystal, F - cut off filter, G - front surface, Q - semitransparent quartz glass, S - sample, PM - photomultiplier, DSA - digital storage adaptor, PC - personal computer, Ti - timer, T- photodiode for external trigger.

(potassium dihydrogenphosphate) crystal to generate the second harmonic in the UV. To obtain a laser pulse with emission wavelengths of 275 nm and 296 nm laser dyes fluorescein (Lambda Physik, FRG, emission maximum 550 nm) and Rhodamine 6G (Lambda Physik, FRG, emission maximum 590 nm) were used. The very short monitoring pulse (ca. 10 ns) and its low power prevented the sample from being damaged by the UV light and a better signal to noise ratio was reached. Two programmable timers (laboratory built) were used, (one in the 0.1-10 μs range and one above 10 μs) to control the time interval between the exciting and the monitoring pulses.

The transmission changes at the given wavelength were detected by two photomultiplier tubes one in front (EMI 9526, GB) and one behind (HAMAMATSU R928, Japan) the sample. The monitoring light was filtered by black UV filters (YΦC5, USSR) behind the KDP crystal and in front of the photomultiplier tubes to ensure that only the UV light was detected. The

photomultiplier signals were simultaneously digitized by a tow-channel digital storage adaptor (THURLBY, DSA 524, GB, 8 bit vertical resolution) and stored in a personal computer.

To check the ability of the system to measure small absorption changes, the absorption of the sample ($A_0=-\lg(I_0/I^0_0)$, where I_0 and I^0_0 are the intensity of the monitoring beam behind and in front of the sample, respectively) was measured, without the exciting laser pulse. I^0_0 and I_0 were determined by averaging 10 measurements. These measurements were repeated 10 times. The dat showed a standard deviation, better than 0.1% which is low enough to allow the desired range of absorption changes (10-50 mOD) to be measured. The absorption changes at 275 nm and 296 nm were also measured at 500 μs after excitation, when they are greatest, to check whether the ratio of the maximal absorption change at 296 nm to the maximal absorption change at 275 nm is approximately 2/3, as reported in the literature (Czégé et al., 1982). For this purpose the absorption of the sample was measured and $A_t=-\lg(I_t/I^0_t)$ has been calculated, where I_t and I^0_t are the intensity of the monitoring beam after excitation behind and in front of the sample, respectively, t=500 μs. The absorption change was calculated as A_t-A_0. I_t and I^0_t were averaged 10 times, and each measurements were repeated 5 times.

The entire kinetics of the absorption changes at 275 nm and 296 nm were then followed, varying the time t, between the exciting and monitoring flashes. Between each set of measurement for a given t the absorption without excitation was measured again.

RESULTS AND DISCUSSION

In Fig. 2 the absorption changes of pm immobilized in polyacrylamide gel at 275 nm and 296 nm are shown. The very good correlation between the kinetic curves obtained using continuous monitoring light as described in ref. Stoylova et al. (1990) (continuous lines), and discrete monitoring pulses (points) for $t > 0.1$ μs (present work) is satisfactory at longer times, and shows that the pulsed method may be used to measure absorption changes of the bR very precisely. Within the first 0.2 μs a very fast decrease in the 275 nm absorption was observed, followed by a slower decrease. This very fast component makes a high relative contribution of about 30% of the total signal. At 296 nm very fast increase of the

absorption was observed similarly. The relative contribution of this very fast component of the 296 nm absorption signal is also high, about 25% (Table 1A). These very fast jumps are attributed to the bR to K transition (about 5ps, not resolved here) (Midler, Kigler, 1988) and could be understood as the aromatic amino acid perturbation near the retinal due to its isomerization upon photoexcitation.

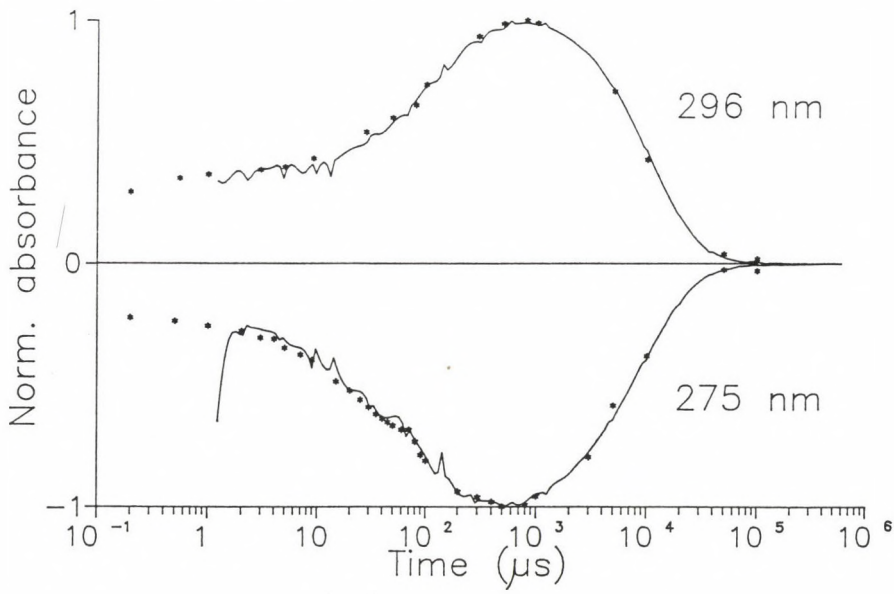

Fig. 2. Comparison of the flash-induced absorption changes at 275 nm and 296 nm, measured with continuous monitoring light (continuous line) and with laser flashes as the monitoring light (points). The absorption curves were normalized.

The life times in the μs range of the different components contributing to the absorption signals at 275 nm, 296 nm, 412 nm and 570 nm are also presented in Table 1B. Two rise components were found in the absorption changes at 412 nm and 570 nm. A very good correlation between the time constants of the fast and slow rise at the two wavelengths was observed. Two components were also found for the 275 nm and the 296 nm absorption changes in the μs range, with slightly slower time constants than those observed in the visible. The fast rising component participates with a low weight in the 296 nm absorption changes at pH7 and low ionic strength and therefore its evaluation by the computer

program was not possible. Its presence had, however, been proved (Hess, Kuschmitz, 1979; Hanamoto et al., 1984). The values of the time constants for the 412 nm and 296 nm absorption changes are in very good correlation to those reported in ref. Hanamoto et al. (1984).

Table 1. Life times and relative amplitudes (in parantheses) of the different components in the μs range, measured with laser flash as monitoring light (A) and with countinuous monitoring light (B). The "?" indicate that the component is not resolved by the computer program.

wavelengths nm	570	412	296	275
life time, μs				
A.			< 0.2 (0.3)	< 0.2 (0.25)
B.	4.9±0.2 (0.25)	5.3±0.5 (0.1)	?	7.5±0.7 (0.15)
	94.5±3.4 (0.5)	91.4±4.9 (0.8)	119±8.3 (0.55)	111±6.1 (0.6)

The same number of kinetic components and their comparable life times, indicate that the events occurring in the chromophore and the protein are reflected in all measuring wavelengths. The slightly slower time constants found in the UV for the slow rising component suggests, however, that first the retinal conformational changes take place, followed by changes of the apoprotein moiety (observed in the UV).

In conclusion, by using noncontinuous monitoring light for measuring the fast absorption changes of the bR in the UV region the earlier steps of the opsin participation in the photocycle can be much better parametrized by their life times and relative contributions to the total absorption changes.

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ANALYSIS OF THE PROTEOLYTIC DEGRADATION PRODUCTS OF HYALINE CARTILAGE PROTEOGLYCANS

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SUMMARY

The proteolytic degradation products of nasal hyaline cartilage proteoglycans produced by polymorphonuclear leukocyte lysosomal enzymes were investigated. The protein content of the degradation products is 7.0 - 8.6 % corresponding to a peptide chain of 24-28 amino acids and the relative molecular mass of the total fragment is M_r=37600 - 39200. On an average, each proteoglycan fragment contains two chondroitinsulphate chains (M_r=22000 - 22400), every fourth fragment contains a keratan sulphate chain (M_r=7000-7200) and every seventh to eighth contains an O-glycosidic oligosaccharide. The results of the disaccharide analysis show that the galactosaminoglycan chains contain 76.2-83.6 % chondroitin-4-sulphate, 12.9-19.4 % chondroitin-6-sulphate, 3.5-3.8 % chondroitin and no dermatan sulphate. Since composition and relative molecular mass of the chondroitin sulphate and keratan sulphate chains from the degradation products resemble those from native proteoglycans, it is suggested that the degradation of the proteoglycans occurs by proteinases that attack preferably the chondroitin sulphate region of the core protein.

INTRODUCTION

Proteoglycans are one of the two major constituents of the articular cartilage matrix. Cartilage destruction in inflammatory joint disease starts with the loss of glycosaminoglycans from the extracellular matrix (Mohr, 1986; Greenwald, 1986). The exact mechanism is still unknown, however proteolytic degradation of cartilage proteoglycans has been considered as well as depolymerisation of hyaluronate. While the importance of proteolytic enzymes secreted by the pannus forming cells in tight contact with the cartilage surface is commonly accepted, there is more controversy concerning the role of proteinases released into the

synovial fluid by polymorphonuclear leukocytes (PMN) (Burkhardt et al., 1987). The PMN are capable of secreting a wide variety of highly destructive neutral proteinases into the extracellular medium, the high levels of proteinase inhibitors have always been considered to be more the sufficient to inhibit the activity of secreted proteinases completely. It is generally agreed that imbalance between proteinase and proteinase inhibitor leads to cartilage tissue breakdown. This may occur after inactivation of the alpha-1-proteinase inhibitor by phagocyte generated oxigen metabolites (Matheson et al., 1981).

Although the degradation of cartilage proteoglycans by isolated proteinases has been studies (Roughley, Barrel, 1977), the exact characterization of the degradation products is still missing. In our previous experiments (Liszt et al., 1991) we developed an in vitro model for the investigation of hyaline cartilage degradation products produced by granule lysosomal enzymes from activated PMN.

In our present paper we describe the exact characterization of isolated degradation products released from hyaline cartilage proteoglycans.

MATERIALS AND METHODS

Chemicals used were analytical grade from Merck, Darmstadt, FRG. Bio-Gel P2 (200-400 mesh), Bio-Sil Amino SS (4 x 250 mm) column were purchased from Bio-Rad Laboratories, Munich, FRG.

TSK SW precolumn, TSK G3000 SW and G4000 SW columns were from Pharmacia-LKB, Freiburg, FRG.

Papain (EC 3.4.22.2) was obtained from Boehringer Mannheim GmbH, FRG. Chondroitinase AC (EC 4.2.2.5), chondroitinase ABC (EC 4.2.2.4), keratanase (EC 4.3.2.1.35) were purchased from Seikagaku Kogyo Co. Tokyo, Japan.

Standard preparations of peptidochondroitin sulphate and peptidokeratan sulphate of known relative molecular mass were described previously (Stuhlsatz et al., 1981).

The procedures for the granulocyte and cartilage preparation and the degradation of its proteoglycans by leukocyte lysosomal enzymes were described in the previous paper (Liszt et al., 1991) together with the isolation and purification of proteoglycan fragments.

Hexosamine and amino acid analysis

Glucosamine, galactosamin and amino acids were determined with a 4151 Alpha Plus amino acid analyser (Pharmacia-LKB). Samples were hydrolysed for 15 hours at 105 °C in 3 mol/l HCl (hexosamines) and for 20 hours at 105 °C in 6 mol/l HCl (amino acids).

Papain digestion

Purified samples of the proteoglycan fragments were treated with papain suspension (100 µg papain/umol hexosamine) in 100 mmol/l sodium acetate buffer pH=6.5, containing 5 m mol/l EDTA.Na_2 and 5 mmol/l cystein hydrochloride for 12 hours. For completeness of digestion, the same amount of papain was added and proteolysed for 12 hours. After papain treatment the peptidoglycan and oligosaccharidpeptide fractions were separated. Dowex 1x2 columns (0.48 x 15 cm) were applied, equilibrated with 0.15 mol/l NaCl. The columns were eluated with 7.5 ml 0.15 mol/l NaCl, 15 ml 0.5 mol/l NaCl and 15 ml 4 mol/l NaCl, respectively. The fractions were desalted on Bio-Gel P-2 column (2.5 x 24 cm) and freeze-dried.

Digestion of the peptidoglycosaminoglycan by chondroitinase AC, chondroitinase ABC and keratanase

Chondroitinase AC treatment: 200 nmol (as hexosamine) portions of peptidoglycane fractions in 50 mmol/l phosphat-buffer pH=6.0 were incubated at 37 °C with 0.5 U chondroitinase AC in the same buffer for 12 hours.

Chondroitinase ABC treatment: 200 nmol (as hexosamine) portions of peptidoglycan fractions in 50 mmol/l phosphat-buffer pH=8.0 were incubated with 0.5 U chondroitinase ABC in the same buffer at 37 °C for 12 hours.

Keratanase treatment: 200 nmol (as hexosamine) portions of peptidoglycan fractions in 50 mmol/l phosphat-buffer pH=7.4 were incubated with 0.5 U keratanase in the same buffer at 37 °C for 12 hours.

Molecular mass determination (M_r) by high performance gel permeation chromatography (HPGPC)

For the determination of M_r TSK G3000 SW (7.5 x 600 mm) and TSK G4000 SW (7.5 x 300 mm) columns with a precolumn cartridge TSK SW5 (7.5 x 75) mm were equilibrated with 40 mmol/l Tris-HCl buffer pH=6.0, containing 0.2 mol/l NaCl, 0.1 % sodium lauryl sulphate. The flow rate was 1.0 ml/min

and the absorbance was monitored at 206 nm. Relative molecular masses (M_r) were calculated on the basis of standard curves, plotted with peptidochondroitin sulphate and peptidokeratan sulphate preparations with known molecular mass (Stuhlsatz et al., 1981; Oeben et al., 1987).

Determination of unsaturated glycosaminoglycan disaccharides by HPLC

Samples with the same hexosamine content after chondroitinase AC and ABC treatment was applied for HPLC. Bio-Sil Amino-5S (4x 250 mm) column with Bio-Sil Amino-5S precolumn (4 x 25 mm) cartridge was equilibrated with 150 mmol/l NaH_2PO_4 buffer, pH=3.9. The flow rate was 1.0 ml/min and the absorbance was monitored at 232 nm. Standard disaccharid kit was used for the quantitative evaluation of 0-, 4- and 6-sulphated disaccharides (Yoshida et al., 1989).

Statistical methods

Significance was calculated using the paired Student's test, accepting statistical significance at the $P<0.05$ level.

RESULTS

The amino acid patterns of the purified proteolytic degradation products of the proteoglycans after 30, 60 and 90 min are very similar (Tab. 1). The most frequent amino acids are Ser, Glu, Pro and Gly in the samples. Quantitative hexosamine analyses show the same galactosamine/glucosamine proportions of 17.0 - 17.4 and the amount of the purified degradation products (measured as galactosamine and glucosamine content) increases from 36.6 µmol to 120 µmol during 90 minutes of incubation time. The carbohydrate and protein content of the proteoglycan fragments could be calculated from the results of their amino acid and hexosamine analysis (Tab. 1). A very important point of this finding is that the fragments have low protein content (7.7-8.6 %), indicating a possibly powerful proteolytic influence.

The relative molecular weight determination shows a very wide peak with average M_r=38200, being evidence of a very heterogeneous proteoglycan population. It could not be observed any differencies in molecular masses during 90 min incubation time (Tab. 2).

Table 1. Amino acid and hexosamine composition of purified proteolytic degradation products from hyaline nasal proteolycans after 90 min incubation time (number of analyses N=5, mean ± S. D., n.s. not significant)

AMINO ACIDS mol/1000 mol	INCUBATION TIME 30 min sample	60 min sample	90 min sample
ASX	59	58	52
THR	53	46	52
SER	150	154	142
GLX	153	149	141
PRO	91	98	100
GLY	157	156	143
ALA	61	69	64
CYSS/2	1	1	1
VAL	52	46	55
MET	5	6	3
ILE	27	28	33
LEU	64	74	83
TYR	30	25	35
PHE	52	47	48
HIS	8	8	8
LYS	15	17	13
ARG	22	18	27
GlcN (µmol)	2.1±0.09	5.0±0.24	6.9±0.35
GalN µmol)	36.6±1.61	84.8±3.99	120.0±6.12
GalN/GlcN	17.4±0.83 n.s.	17.0±0.87 n.s.	17.3±0.86
Amino acid (µmol)	16.6±0.80	35.5±1.85	44.4±2.75
Protein %	8.6±0.39	7.9±0.38	7.0±0.35
Carbohydrate %	91.4±4.11 n.s.	92.1±4.51 n.s.	93.0±4.68

For the characterisation of the glycosaminglycan-chains in proteoglycan degradation products the 30, 60, 90 min samples were proteolysed by papain. After papain treatment the peptidoglycans and oligosaccharide peptides were separated by Dowex chromatography.

Table 2. Average relative molecular masses (M_r) of proteoglycan fragments and glycosaminoglycan chains, structure of proteolycan fragments released from nasal cartilage (number of analyses N=5)

	INCUBATION TIME		
	30 min	60 min	90 min
M_r total	38300	39200	37600
$M_r$chondroitinsulphat	22400	22400	22000
$M_r$keratansulphat	7200	7200	7000
KS chains/proteoglycan fragment	0.24	0.23	0.24
O-linked oligosaccharides/ proteoglycan fragment	0.13	0.13	0.13
Chondroitin	3.5 %	3.8 %	3.8 %
Chondroitin 4-sulphate	83.6 %	83.1 %	76.2 %
Chondroitin 6-sulphate	12.9 %	13.1 %	19.4 %

The M_r values of the peptidoglycan fractions were determined on a TSK G3000 SW column (Fig. 1) and the average M_r of two populations (peak 1 and 2) are M_r=22400 and M_r=7200, identical for the samples 30, 60, 90 min (Tab. 2). In order to identify the glycosaminglycan chains of the fragments, the peptidoglycan fractions were digested by chondroitinase AC, chondroitinase ABC and keratanase. After the enzymatic treatment, according to the HPGPC analyses on a TSK G3000 SW column, peak 1 could be identified as chondroitin sulphate and peak 2 as keratan sulphate population (Fig. 1).

In addition to the digestion of peptidoglycan fractions with chondroitinase AC and ABC, the determination of unsaturated disaccharides yielded from chondroitin, chondroitin 4-sulphate and chondroitin 6-sulphate was performed. The results of disaccharide analyses show (Tab. 2) that the galactosaminoglycan chains contain in all samples 76.2-83.6 % chondroitin 4-sulphate, 12.9-19.4 % chondroitin 6-sulphate, 3.5-3.8 % chondroitin and no dermatan sulphate.

DISCUSSION

In previous years, interest has been focused on the cartilage matrix degrading potential of lysosomal enzymes (Schalkwijk et al., 1987; Barret, 1978; Velvart et al., 1981), such as elastase, metalloproteinase, cathepsin G etc. Although the studies are quite informative on the degradation of proteoglycans by isolated proteinases (Roughley, 1977; Schalkwijk et al., 1988), the characterisation of the degradation products is still missing. In the present study the isolated proteolytic degradation products of hyaline cartilage proteoglycans were analysed.

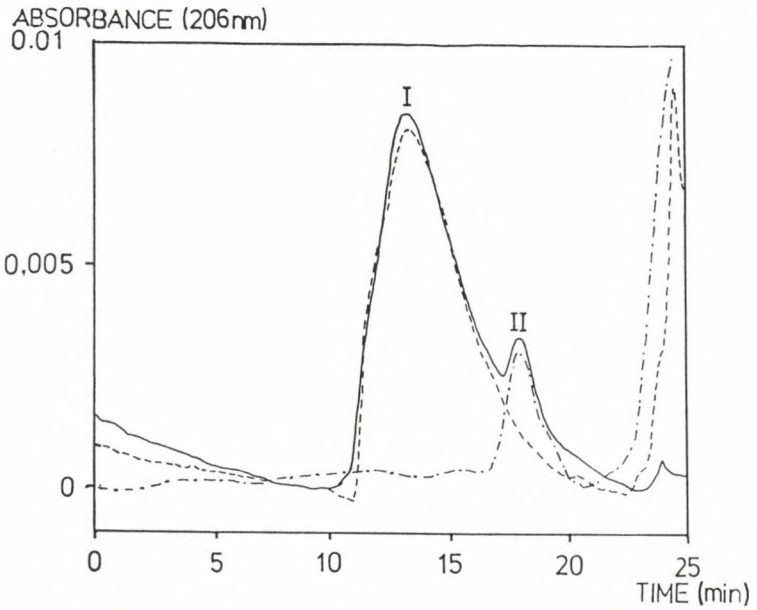

Fig. 1. Molecular-size profiles of the peptidoglycosaminoglycan fraction after papain treatment (——) and chondroitinase AC (- · -) or keratanase (--) degradation chromatographed on TSK G3000 SW. Peak I: chondroitin sulphate, peak II: keratan sulphate.

The amino acid analyses for the purified proteoglycan fragments demonstrated the same distribution of amino acids, serine, glycine, glutamic acid and proline were the most abundant amino acids indicating a very distinct proteolytic attack near the chondroitin sulphate-peptide linkage region. The protein content of the proteoglycan fragments is 7.0-

8.6 % corresponding to a peptide chain of 24-28 amino acids and the relative molecular mass of their carbohydrate moiety is M_r=37600 - 39200. After digestion with papain the oligosaccharide moiety was separated from the glycosaminoglycans and their relative molecular mass was determined resulting two mean peaks with M_r=22000-22400 and M_r=7000-7200. They were identified by degradation with keratanase and with chondroitinase AC and ABC. Thereby the population with an average molecular mass of M_r=22000-22400 was related to chondroitin sulphate and that with M_r=7000-7200 to keratan sulphate. From the quantitative hexosamine analyses in the oligosaccharid peptide fraction and from the number of glycosaminoglycan chains the number of o-glycosidic oligosaccharides could be calculated.
Our results demonstrate (Tab. 2) that on an average, each proteoglycan fragment contains two chondroitin sulphate chains, every fourth a keratan sulphate chain and every seventh to eighth an O-glycosidic oligosaccharide and we could not detect any N-glycosidic oligosaccharide. The results of our disaccharide analyses show that the galactosaminoglycan chains contain mainly chondroitin-4-sulphate and chondroitin-6-sulphate, only 3.5-3.8 % chondroitin and no dermatan sulphate.
Since composition and relative molecular mass of the chondroitin sulphate and keratan sulphate chains from the degradation products glycan fragments resemble those from native proteoglycans (Poole, 1986), the conclusion may be drawn that the degradation of the proteoglycans occurs by proteinases alttacking preferably the chondroitin sulphate rich region of the core protein.
It was shown that lysosomal enzymes (Roughley, Barret, 1977; Roughley, 1977) are able to degrade cartilage components such as proteoglycan and collagen. Two PMN neutral proteinases, elastase and cathepsin G were identified as potent degradative agent. Roughley (1977) found that the final proteolytic degradation products of the isolated proteoglycans are clusters with different glycosaminoglycan chain number. The initial cleavages occur at sites that are common to the majority of proteinases. Further cleavages occur within the groups, in peptide regions whose susceptibility to proteolysis is more dependent on the specificity of the proteinase. Such cleveage may lead to the production of cluster that cannot be degraded further by the proteinases. Under in vivo conditions the degradation occurs in the synovial system, the morphological and biochemical unity of cartilage, synovial fluid and lining cells. In

normal cartilage most of the large proteoglycans are bound to a single hyluronan molecule, being stabilized by the socalled link protein. Cleavage of the hyaluronan chain leads to low molecular hyaluronan that is no more able to form aggregates. The proteinases of PMN, i.e. elastase, metalloproteinase, cathepsin G, are able to degrade the core protein of the proteoglycans (Greiling, 1986). This leads to the formation of low molecular fragments (glycosaminoglycan peptides) that are more water-soluble and therefore will be extruded from cartilage into the synovial fluid.

Acknowledgement

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THE EFFECT OF 2.45 GHz MICROWAVE IRRADIATION ON HUMAN PERIPHERAL LYMPHOCYTES

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SUMMARY

The effect of 2.45 GHz frequency and 10 mW/cm^2 power density microwave irradiation on the in vitro cultured human peripheral lymphocytes blast transformation was examined. In the number of the blast cells we did not find significant difference at cultures irradiated for 1 x 5 hours and 3 x 1 hours as compared to the controls. The number of the blast cells was found to increase when Phytohemagglutinin-stimulated lymphocyte cultures were exposed to repeated irradiation for 6 days (1 hour a day). The microwave irradiation did not influence the degree of the spontaneous blast transformation in any groups.

INTRODUCTION

In the second part of this century, in consequence of the progress of microwave technique, the equipments of telecommunication, therapeutic devices and household gadgets using microwave radiation became general all over the world. The quickly increasing number of microwave sources turned the attention to the biological aspects of radiation induced by them. The existence of certain biological consequences is proved by epidemiological surveys, which studied subjective complaints (headache, tiredness, etc.), and by experimental results of biochemistry, cellbiology, genetics, immunobiology and neurobiology (Polk, Postow, 1986; Repachoti, 1988; Predmerszky et al., 1982). Hitherto research has revealed several basic features of the biological effects of microwave radiation, such as trigger character and frequency dependence (Czerski, 1975), and referred them to the role of physiological condition and irradiation circumstances (Michaelson, 1983). The possibility of carcinogenic late effect was also pointed out.

In a large number of countries, as also in Hungary, safety standards were published for the limits of this type of non-ionizing radiations to preclude first of all the possibility of thermal damage.
However, an unsettled question is the existence of non-thermal effects, which are not attributed to temperature rise caused by the energy absorption. The biological consequences of long-term irradiation with low power density (e.g. specific absorption rate, SAR) are almost unknown.
Information accessible by sensitive immunological tests, for example the study of the blast transformation of peripheral lymphocytes, may contribute to the answer. Unfortunately, there is some contradiction among the few human examinations in this field (Doronov et al., 1968). In order to make the problem more clear we investigated spontaneous and PHA induced blast transformation of human peripheral lymphocytes exposed to 2.45 GHz microwave radiation.

MATERIALS AND METHODS

Blood samples were obtained from 29 healthy men, mean age 20 years (range 19-22). Heparinized whole blood was diluted with Eagle Modificated Eagle Medium (E.MEM, OKI) 10 times and the suspension was completed with 5% fetal calf serum (FCS, Phylaxia).
The blood samples were divided into 3 portions. To the first portion Phytohemagglutinin-P (PHA, Difco) was added to a final concentration of 5 ug/ml in 10 ml total volume of cell suspension at the start of the culture (PHA treated control). The 2nd portion was exposed to microwave while the 3rd portion of the samples was treated both ways.
The cultures were incubated at $37^{o}C$ in an atmosphere of 5% carbon-dioxide. After the incubation times of 3 or 6 days the lymphocytes were prepared as follows: the cultures were centrifuged for 10 minutes at 500 x g and the pellets were suspended with 7.5 mM KCl solution for 6 minutes in order to hemolyze them. Thereafter, the cultures were washed and fixed with methanol:acetic-acid mixture (3:1). The cells were dropped on microscope slides and were stained with Giemsa-solution.
The results were obtained after morphological observation on hemocytometer. The normal lymphocyte in blood has a nucleus of 4 to 6 µm in diameter. After the addition of PHA, the lymphocyte enlarges and becomes to acquire basopholic cytoplasm and prominent nucleus. Large

lymphoid cells of more than 7 μm in diameter on the hemocytometer were defined as PHA-reacted blast cells.

Continuous wave irradiation of 2.45 GHz frequency and 10 mW/cm^2 power density was carried out in a Faraday-room in the near-field zone of a horn antenna. The specific absorption rate (SAR) was 14.8 mW/g in the blood samples.

We used 3 different ways of irradiation: one single bout of 5 hours or 1 hour/day repeated irradiations lasting for 3 or 6 days. During irradiation the temperature of the cultures was 36 ± 1°C.

RESULTS

In the unstimulated control cell cultures the spontaneous blast transformation was very low, on the 3rd and 6th day the percentage of the blast cells was 8.6 ± 6.3% and 4.2 ± 3.8%, respectively.

Table 1 indicates the percentage of the blast cells on the 3rd day in 10 different cultures after PHA treatment and/or for 5 hours irradiation immediately at the start of the incubation. The data show a little decrease in the number of blast cells in PHA and microwave treated cultures as compared to the PHA treated controls.

Table 1

Percentage of blast cells in 3-day-old whole blood samples treated with PHA and/or 1 x 5 hours microwave irradiation at the beginning of incubation

Sample number	PHA treated (%)	Microwave irradiated (%)	PHA treated and irradiated (%)
1	55.0	8.4	60.6
2	60.5	7.6	66.7
3	78.2	7.2	72.0
4	77.7	10.3	65.3
5	68.5	10.3	72.1
6	54.0	5.0	53.0
7	51.2	4.4	38.0
8	46.8	9.5	39.8
9	45.9	5.6	30.2
10	44.6	6.1	29.4
Mean	58.2	7.4	52.7
SD	12.6	2.2	17.0

In order to examine the effect of the repeated irradiation we applied irradiation through 3 or 6 days (one hour/day) on each cell culture of 9 and 10 persons, respectively. The first irradiation was applied on the first day of the cell culture and the followings were carried out in the consecutive 24 hours. The results are indicated on Table 2 and 3. Although there was no marked effect at the samples irradiated 3 times (Table 2), the 6 times repeated irradiation caused a significant increase in the number of PHA induced blast cells compared to the PHA treated controls (Table 3).

Table 2

Percentage of blast cells in 3-day-old whole blood samples treated with PHA at the beginning of incubation and/or exposed to 3 x 1-hour repeated microwave irradiation

Sample number	PHA treated (%)	Microwave irradiated (%)	PHA treated and irradiated (%)
1	60.4	3.7	49.2
2	59.2	2.2	59.0
3	76.5	10.5	38.5
4	70.0	17.5	67.1
5	48.0	4.3	45.8
6	68.3	5.3	58.8
7	51.3	17.3	70.3
8	67.5	10.3	59.8
9	68.3	11.0	74.0
Mean	63.3	9.1	58.1
SD	9.3	5.7	11.7

All of these results seem to be supported by statistics (Table 4). We found significant difference at the PHA treated groups only in case of 6 times repeated irradiation. It can be proved, that the 2.45 GHz frequency microwave radiation did not have any effect on the spontaneous blast transformation of in vitro cultured human peripheral lymphocytes.

Table 3
Percentage of blast cells in 6-day-old whole blood samples treated with PHA at the beginning of incubation and/or exposed to 6 x 1-hour microwave irradiation

Sample number	PHA treated (%)	Microwave irradiated (%)	PHA treated and irradiated (%)
1	41.8	4.3	68.7
2	39.7	5.5	50.5
3	43.3	10.3	75.8
4	25.3	2.4	66.5
5	37.5	4.5	67.0
6	47.2	5.2	65.5
7	28.7	4.8	58.9
8	46.9	9.0	72.3
9	49.4	6.5	77.8
10	46.5	2.6	75.8
Mean	40.6	5.5	67.9
SD	8.1	2.5	8.4

Table 4
Significance levels computed by one-tailed t-test

Groups compared	Type of irradiation	Significance level
PHA treated compared to PHA treated and microwave irradiated	1 x 5 hours	$p < 0.073$
	3 x 1 hours	$p < 0.34$
	6 x 1 hours	$p < 0.000002$

DISCUSSION

In recent years many articles have been published about the non-thermal influence of the microwave radiation on the immune system. There is still no consensus about either the existence of this effect (Smialowicz, 1976, 1979) or whether the proposed effect should be expected to stimulate (Grundler et al., 1977; Ivanoff et al., 1979; Robert et al., 1978; Schlagel et al., 1979; Wiktor-Jedrzejczak et al., 1980) or suppress (Czerski, 1975; McRee et al., 1979; Szmigielski et al.,

1980) the immune system. Moreover, it causes complication, that the tendency and the degree of this alteration depends on several parameters (field-intensity and frequency, duration and exposition frequency, physiological state, etc.). We found significant increase in the percentage of blast cells in samples being irradiated for 6 days.

For explanation of this difference it can be concluded, that microwave radiation has real effect on blast transformation, increasing the number of blast cells, but this manifests later. Another interpretation is that microwave radiation, slowing down the biological processes, prolongs the life-time of the cells being divided and this causes the greater number of the blast cells. This is supported by Fröhlich's hypothesis (Fröhlich, 1980) and some other experimental results (Grundler et al., 1977), which predict the temporal holdup of the dividing processes during and after the microwave irradiation. Because the majority of the cells of the 6-day-old culture treated with PHA got over blast transformation and died, the percentage of the blast cells in these cultures is low ($40.6 \pm 8.1\%$ against the $63.3 \pm 9.3\%$ and $58.2 \pm 12.6\%$ in the 3-day-old samples). It can be concluded that the cultures irradiated by microwave for 6 times 'survived' better than the PHA treated controls and showed greater number of blast cells.

It is worth noting, that after in vitro PHA stimulation the dividing T-lymphocytes could be classified into 2 groups: a late and a fast type in division (Onody, 1978). It could be imagined, that microwave radiation has a selective effect on one of the groups and causes the above-mentioned relative increase in the number of the blast cells on the 6th day.

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111-INDIUM HUMAN ANTIMYOSIN MONOCLONAL ANTIBODY-FRAGMENT STUDY ON ALCOHOLIC (AND DIABETIC) CARDIOMYOPATHIC PATIENTS (ACM-DCM) WITH CENTOCOR EUROPE MYOSCINT (Msc)

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SUMMARY

The criteria of Msc-positivity were: typical accumulation of hot spots in 2 directions of planar scan in the 24-48-hr measurements, while in atypical cases the hot spots were present only in the 48hr picture.

Twenty-one male volunteers were assessed. 18 of them had ACM (2 patients were studied repeatedly). The negative and positive control cases exhibited the expected Msc pattern. Seven ACM patients were regarded typically positive, in 5 cases atypical positivity was stated, and 6 patients were classified as Msc negatives. The heart lung activity ratio was 1.75 ± 0.45 and 1.68 ± 0.44 for typical and atypical positive cases, respectively. The control and DCM H/L ratios were 1.15 and 1.3 ± 0.2, respectively, in AMI the H/L was 1.91, in DCM of dilatative phase it was 1.55 ± 0.06 with serum myosine in the normal range. Heart dilation in the DCM groups: 5 dilated out of 7 typically positive cases while in atypically positive, and negative cases the dilation occurred in 2 out of 5 and 1 out of 6 cases, respectively. Low LV-EF (less than 45%) were found in ratios of 4/7, 2/5, and 1/6 in typically positive, atypically positive and negative cases, respectively. Elevated PCP similar ratios. In the same groups pathological ECG was seen in ratios of 5/7, 1/5, and 1/6, respectively. The g-GT activity was enhanced in all cases, there was, however, no evident correlation between the actual enzyme activity and intensity of hot accumulation.

The 4 typical and 2 atypical positive cases with diffuse patchy Msc uptake were patients in progressive stage of the ACM or DCM, with heart dilation, rhythm disturbance. The Msc positivity of the ACM and DCM patients seem to be not specific, but the consequence of progressive dilatative phase of their cardiac illness. (Autoimmune carditis may perhaps play some role in this.)

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INTRODUCTION

The problem of structural and functional integrity of the heart is equally important in the everyday practice and in research. Cell death goes together with the derangement of membranes so intracellular elements leave the cell. Being the covalent structure of the myocardial myosin unique, the application of antimyosin (or the Fab'-fragment) could be a good marker of a myocardial lesion.

The first human studies by the Research Group of Khaw, Beller, Haber and Smith (1976) were directed to the detection and localisation of the acute myocardial infarction. Later the studies were extended to a number of diagnostic problems, like the noninvasive follow-up of heart transplant patients (Carrio et al., 1989), or the acute myocarditis (Haber et al. 1987), but the ACM and DCM were still not among them (Berger et al., 1986).

The aim of our study was therefore to examine the structural and functional heart disorders of heavy drinkers and of diabetics, being affected mostly by ACM and DCM, respectively.

METHODS

The antimyosin Fab'-fragment Myoscint (Msc, lot 87G 2101), produced in mice against human myosin was received as a support of Centocor Europe. The bifunctional chelate DTPA served for covalent linking of the 111-In. The Msc and the DPTA charges were the same in all experiments. The requirements of successful labelling were known from the quality control experiments of Haukka and Hiltunen (1987). The radiochemical purity-quality of the used Amersham 111-In radionuclide was examined and found suitable by the Ge/Li detector of the Veszprém University of Chemical Engineering. We detected the peaks 23.2 keV of Cd-$K_{alpha-1}$, 171.29 of 111-In. We used the photopeaks of 171 and 245 keV-s. The yield of the 111-In label was always more than 90 %. Control R_f-values (Msc, DPTA, $InCl_3$) agreed with the literature data. Clinical side effects of the radiopharmacon were not observed, even after repeated application.

Khaw and coworkers (1986) published human Msc-investigations with 99m-Tc labelled antimyosin, too, but this labelling is more complicated than labelling the 111-In and the method is being only in its experimental phase.

The criterion of Msc-positivity was the accumulation of hot spots in the 24-48hr samples from al least two detected directions. If accumulation was seen in the 48hr sample only or in only one direction, it was regarded as atypical positivity.

The investigations were carried out using a Hungarian scintillation gamma camera with built-in dedicated computer (Gamma Works MB 9100-9101, Hungary), by means of planar technique. The documentation was printed out and taken on color polaroid.

The applied methods and investigation strategy are listed in Table 1. We held the injected dose as low as possible, the activity was 2mCi-74MBq for each radioisotope or radiopharmacon. In the case of 99m-Tc and 123-I the thyroid uptake was blocked, the half-lifes of the 99m-Tc, of 113m-In and of 123-I are short, so their radiation doses were relatively low.

The critical organ for 111-In Msc is the kidney with 8.8 rem) 2mCi dose. The radiation burden for the whole body 1.02 rem/2mCi, the load of liver, spleen, bone marrow and gonads are in the 1-2 rem/2mCi range.

The investigations were carried out according to international routine standard procedure, - the details are described elsewhere (Horváth et al., 1984; 1987a; 1987b). As regards the 123-I heptadecanoic acid tool for studying myocardial accumulation and metabolism of the free fatty acid, we followed the instructions of Feinendegen and coworkers (1981). The radiopharmacon was obtained from Squibb-vanHeyden. For approximate quantification of the Msc hot spot, we calculated the heart/lung quotient, compared with proportional cut off ratios of normal subjects as published by Carrio and coworkers (1988). Otherwise we utilized the experience of our Finnish research partners Kuikka and coworkers (1987) accumulated by parallel double 111-In Msc and 99m-Tc pyrophosphate (SPECT) measurements in myocardial infarctions.

Patients

Eighteen ACM patients were studied, mostly middle aged (40-60yr) and moderately obese men exceeding the norm by 10 to 20 kg. An acute transmural posterolateral myocardial infarction patient with reinfarction in the anteroseptal region and another patient with autoimmune myocarditis complicated extensive myocardial infarction in the dilatative

congestive stage served as positive controls and one healthy subject as negative control.

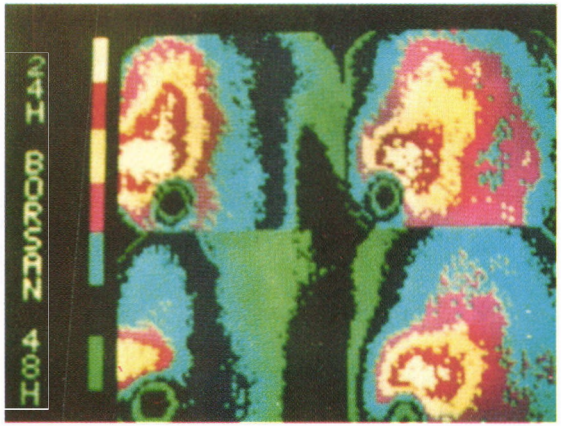
The quantity of alcohol consumption was more than 100 g daily even at the time of the investigation, - previous detoxication cures had been unsuccessful. For characterization of the acute situation we measured the serum gamma-glutamyl transpeptidase activity.

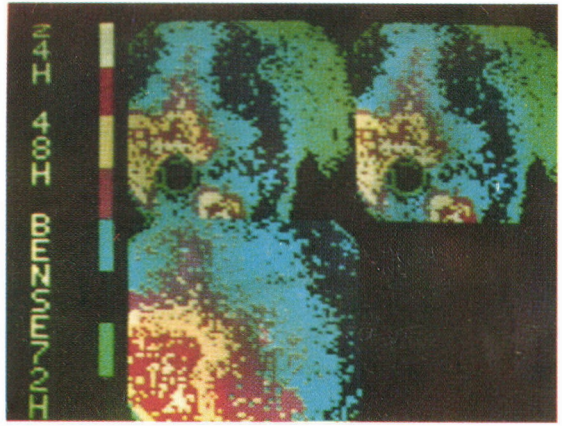
The course of the illness in 11 middle aged, slightly overweight diabetics was more than 10 yrs. Two patients were treated with insulin. The insulin levels (measured by RIA) were 34 μU/L for males and 26 μU/L for females. One male and 3 females had myocardial infarctions previously, one with complicating cardiac aneurysm.

RESULTS

By the Msc method, our acute myocardial infarction (AMI) patient, on the 7^{th} day of his illness, had hot spots in posterolateral localisation, with a heart/lung ratio of 1.91. According to Lahiri and Coworkers Msc, in contrast to 99m-Tc pyrophosphate can provide diagnostic images up to the 10^{th} week post MI (Jain et al., 1989). This is concordant with the observation that elevated serum myosin levels (examined by RIA) can still exist at the end of the 1^{st} week of infarction (Katus et al., 1984). The Msc hot spot over lapped the pyrophosphate spot and was congruent with the 201-Tl defect also observed in the AMI patient. The MI patient with postinfarction dilatative and congestive cardiomyopathy had, according to the immune reaction, diffuse uptake of similar intensity. The heart/lung ratio of the normal subject was 1.15.

By the criteria applied in our practice (the hot cut off ratio at about 1.3-1.4) 7 examinations including the 2 repeated ones were classified as ones yielding typical positive results, and 5 gave atypical positive results. From the comprehensive clinical table it is obvious that the patients with heart dilatation, rhythm disorder, diminished left ventricular ejection fraction and increased pulmonary capillary pressure belonged to the "typical positive" group. From Table 2 it is also obvious that there was not unequivocal correlation between the actual g-GT activity and the heart/lung ratio of the ACM cases. The 123-J heptadecanoic acid kinetics was equally pathological in all cases studied, independent of the Msc positivity. This was not unexpected because of the known severe disorder of lipid metabolism in alcoholics.

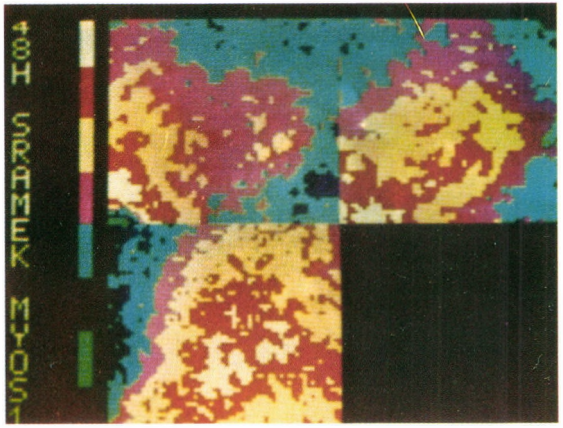


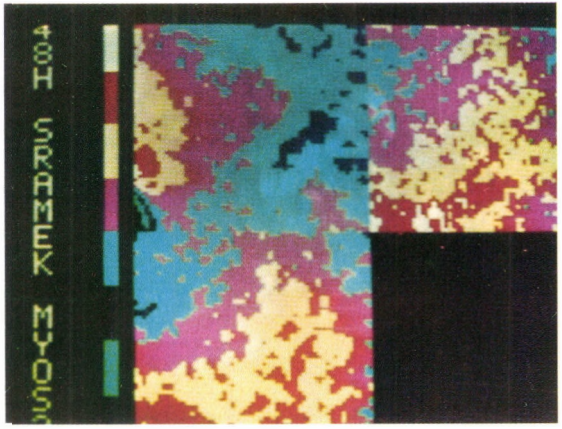


24-48 hrs
AP-LAO 45°

CONTROL PERSONS
heart area free
activity only
in the sternal and liver
regions

24-48-72 hrs
LAO 45°





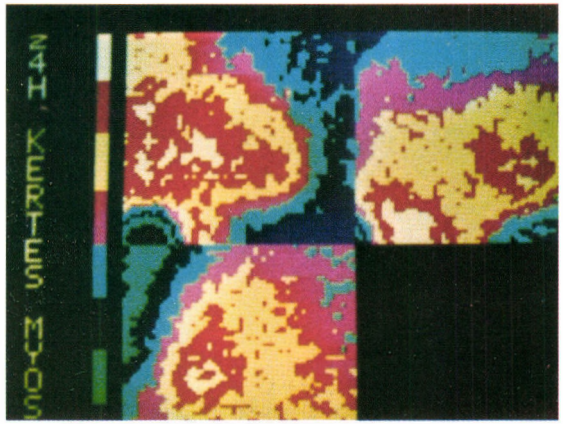
heart /lung
Q 1.54 LAO 45°
N.S. 46 yr
Cardiomyopathia
alcoholica
Obesitas. Hypertonia
Cardiodilatation
LV-EF 51%
PCP 11 Hgmm

REPEATED MYOSCINT
after 1/2 yr.

201-Tl
scan
ECG
Liver
scan

48 hr POS heart/lung
AP-LAO 45°-LAO 70° Q 1.72

ergometric stress
septal hypoperfusion
left strain
indirect cirrhotic signs
γ-glutamyl transferase act
88 U/l



atypical hot accumulation

24 hr.

G.K. 57 yr

Dg. Cardiomyopathia alcoholica

Obesitas. Hypertonia

Hyperlipaemia

Cardiodilatation

LV-EF 13%

PCP 35 Hgmm

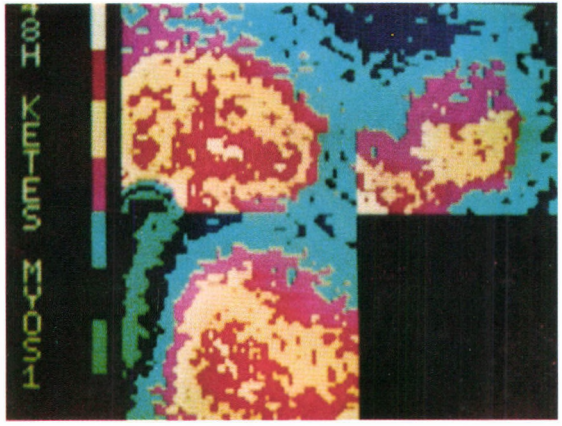
ECG ischaemic pattern

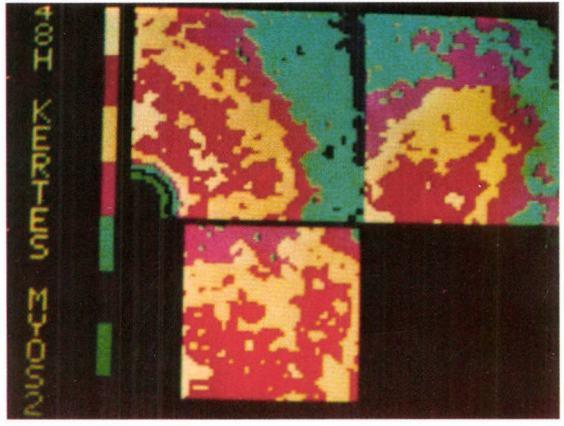
201-Tl myocardial perfusion

permanent ant. hypoperfusion

γ-glutamyl-transferase 162 U/l

act.





48 hr
MYOSCINT
typical positive heart/lung
Q 1.47
1st exam

repeatedly typical positive
after 1/2 yr heart/lung
Q 1.93

Fig. 1. Typical Msc positivity, given in time course
The hot uptake extends to the whole heart with
patchy character

We confirmed the observations of Bridgen and Robinson (1964) that a positive liver scan of the ACM is not obligatory, the lesions of the heart and the liver are not running parallel. Overlap with 201-Tl scan in this age group and with severe heart disorder can occur.

Figure 1 typical Msc positivity, given in time course. The hot spots extend to the whole heart, giving a patchy character of the picture.

Similar was the case in DCM patients. The 48 hr heart/lung ratio for males and females was 1.38 ± 0.04 and 1.33 ± 0.11, respectively. Separating the 3 males and 2 females with dilatative CMP, the ratio became 1.55 ± 0.06 for others it was 1.46 ± 0.13. The control ratio of this new experiment was 1.3 ± 0.2.

In addition, we determined the serum myosin level of DCM patients by Pasteur Diagnostics RIA kit (beta type heavy chains), and found that it was in the normal range, under 100 ng/ml.

DISCUSSION

The myosin isoenzyme distribution of the venctricular muscle has been documented in animal experiments in different cardiac diseases (Édes et al., 1984). Strong indirect immune-fluorescence reaction has been obtained in biopsy material of alcoholies (Hőgye et al., 1988). The immune mechanism may be accompanied by carditis with Msc positivity of diffuse or patchy character. Neu suggested the myosin as cardiac antigen (Neu, 1988).

Our opinion is that the Msc positivity of ACM is not specific, it is rather a consequence of progressive, dilatative CM. This has been supported by the data of the diabetic progressive cardiomyopathics. (They suffer not only from ischemic coronary heart disease, but from micro-angiopathy of the heart, as well. In ACM the symptoms of myocardial derangement have been verified together with an incongruence between the hot spot intensity and the actual g-GT level. Since our first publication (Horváth et al., 1989), Munz and Coworkers documented Msc positivity in ACM (Munz et al., 1989). They maintain that the histomorphologically verified interstitial fibrosis reflects repair mechanism to myocardial injury. Therefore repeated investigations are very important, but care must be taken because of possible immune reaction of Msc. The Msc scan is very promising in checking the salvage of myocardium after coronary reperfusion (Sochor et al., 1987).

We are grateful to Centocor Europe for providing the Msc kit free of charge.

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Table 1

List of methods and investigation strategy

CATEGORISATION STRATEGY OF THE MYOSCINT STUDY PATIENTS

CLINICAL DIAGNOSIS

ALCOHOL CONSUMPTION - LIVER SCINTIGRAPHY
g-GT, GOT-ASAT, GPT-ALAT
serum bilirubin

AGE PLUS BODY MASS BLOOD PRESSURE

ELECTROCARDIOGRAPHY basal and stress

201-TL MYOCARDIAL PERFUSION basal and redistribution

123-I HEPTADECANOIC ACID myocardial accumulation and
metabolic utilisation kinetics

MYOSCINT

111-IN MYOCARDIAL NECROTIC HOT SPOT

99m-TC PYROPHOSPHAT MYOCARDIAL NECROTIC HOT SPOT

113m-IN or 99m-TC RADIOCARDIOGRAPHY-VENTRICULOGRAPHY
CO, SV, LV-EF basal and stress
PCP (RKG derived)

99m-TC RBC CARDIAC BLOOD POOL SCINTIGRAPHY - ECHOCARDIOGRAPHY
regional LV wall movement

CONTRAST VENTRICULOGRAPHY

LEFT HEART CATHETERIZATION

RIGHT HEART MICRO-CATHETERIZATION

MECHANO-CARDIOGRAPHY

The investigations were carried out according to international routine standard methods.

Table 2
Comprehensive presentation of the results (classical clinical results, ECG, nuclear cardiology and medicine, serum gamma glutamyl transpeptidase activity)

	MYOSCINT positive typical	MYOSCINT positive atypical	MYOSCINT negative	normal control	AMI control
	7	5	6		
Hot (spot) accumulation heart/long ratio	1.75±0.45	1.68±0.44		1.15	1.91
	48 hrs	24 hrs		48 hrs.	
(left)-heart dilatation	5/7	2/5	1/6		
LV-EF 113m-In -55reb % transferrin RKG 99m-Tc RBC RNV	1/7	2/5		(60 ± 5%)	
45-55	2/7	1/5			
30-45	2/7	1/5			
15-30	-	1/5			
15-	2/7				
Pulmonary capillary pressure (derived from RKG) more than 15 Hgmm	3/7	1/5	1/6	(less than 12 Hgmm)	
E C G					
only basal normal	3/7				
stress normal		2/5	1/6		
stress ischaemic	4/7	3/5	5/6		
rhyth. disorder	1/7	1/5	1/6		
201-Tl myocard perfusion scan					
normal	2/7		1/6	(regional activity defect	
ischaemic redistribution	1/7	2/5	4/6	more than 20 %)	
permanent	2/7	2/5	1/6		
ischaemic+permanent	2/7	1/5			
123-I heptadecanoic acid scan and kinetics deficit and metabolic delay	1/1	1/1	3/3	(similar as 201-Tl defect: fatty acid kinetics t 1/2 more than 32 min)	

Liver scan activity defect	5/7	1/5	4/6	over 20 mm ϕ
99m-TcFyton, 111-In Msc., resp.				
Serion gamma-glutamyl transpeptidase	153±155	259±148	172±141	(less than 40 μ/l)
g-GT activity				
-100 μ/l	4/7		1/6	
100-300	2/7	2/5	3/6	
300-500	-	1/5	2/6	
500-	1/7	2/5		

123-1 heptadecanoic acid investigation was performed only in some cases, but it was always "positive".
THE MYOSCINT AS MONOCLONAL HUMAN ANTIMYOSIN-FRAGMENT IS THE PRODUCT OF CENTOCOR EUROPE

EDUCATION OF BIOPHYSICS

Acta Biochim. Biophys. Hung. 25/3-4/, pp. 177-178 /1990/

INTRODUCTION

Education creates the most important basis of the future development of all disciplines. This opinion gave the iniciative to continue the tradition of the Special Commission on Education and Development in Biophysics of the International Union for Pure and Applied Biophysics. Joined to the 9th International Biophysics Congress, August 23-28, 1987 Professor Fuller organized the IInd Panel Forum on the problems of education of biophysics. The Ist Panel Forum was held in Mexico City, in August 1981, which was published in 1982.
The IInd Panel Forum included presentations from various geographic areas of our Globe.

Unfortunately, collection of manuscripts took a long time, but the editors believed that the principle "better late than never" is valid in this field, too.

We hope that this booklet will be useful for every university, where a terciary or quarternary education of biophysics is conducted, perhaps its importance for developing countries will be even greater.

We should like to draw the attention of professors of biophysics for a contradiction, i.e. an increase of biophysics teaching units of the universities does not correlate with an increase of biophysical research laboratories. We should like to quote Prof. H. Eisenberg who applied - with his usual witticism - Moliere's humorous statement to the situation of biophysics at the opening of the IXth International Biophysics Congress in Jerusalem. "Many scientists conduct biophysics without beeing

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aware, that they are doing biophysics". Really, the discipline of biophysics is taught at numerous universities in the frame of general physiology, medical physics, medical electronics, neurobiology, crystallography etc. We believe, that all these disciplines can use the information collected in this booklet.

The editors are grateful for the Hungarian Academy of Sciences for the generous support of publication.

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WHAT ARE THE BASIC CONTENTS OF GENERAL BIOPHYSICS?

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Biophysics is a rapidly developing interdisciplinary science which in addition to being an important branch of biology has become a proper discipline in its own right. We might say that without biophysics, biology cound not be prefect. Biophysics helps us to uncover the nature of life and to understand the underlying mechanism of living phenomena.

Biophysics is a discipline which has a powerful 'cohesive force' because not only can it draw the branches of biology together, but it can also draw together the following four disciplines: biology, physics, chemistry and mathematics. There are two peculiarities in the way science is currently developing. First, the methods and techniques used for researching in one discipline are commonly used in other disciplines. Second, the boundaries between disciplines are diminishing. Since biophysics is at the interface of all the branches of biology and of all the other natural sciences with biology, all these other natural sciences can be expected to find a place within them for techniques and concepts from biophysics.

Are there any other peculiarities of biophysics in general? We would like to mention the following. Biophysics is a discipline which can unite experimental and theoretical considerations: It can also unify microscopic and macroscopic views: other words, it can unify the molecular level and the whole organism level. As biophysics is a science developed to deal with complicated systems, it can unite the study of matter, energy and information. Branches of biology other than biophysics can deal with only one or two of the above three quantities, but biophysics has the potential to deal with all three quantities as a whole. If it has not been done yet, I think it will probably be done in the future.

Why emphasise these three quantities? We know that the nature of

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life is living. But what is living? We may say that living is the comprehensive appearances of the three quantities: matter, energy and information, which means that these three quantities are continuously changing but the activities of organisation and ordering of these three quantities is the basis of life. Of course, in living systems, the movement and change of these three quantities are very complicated and very rapid. Now we can measure ultrafast reactions, so we have more knowledge of living systems. As techniques develop we acquire more and more knowledge about the correlations of these quantities. As living organisms develop, we see that biomatter is more and more complicated, the utilisation of energy is more and more delicate, and the information quantity increases more and more. If we research life, we must research these three quantities, and research their correlations and the movement and development of these three quantities.

Before we duscuss the basic contents of general biophysics we should answer the following three questions: (1) what is biophysics? (2) what are the aims of biophysics teaching and (3) to whom is biophysics to be offered.

The question 'What is biophysics' is still difficult to answer simply and this paper is not intended to discuss this subject. I would, however, like to say that I myself would be subject. I would, however, like to say that I myself would be inclined to accept Hill's definition of biophysics. . . "Biophysics is the study of biological function, organisation and structure by physical and physiochemical ideas and methods". Secondly, the aims of general biophysics teaching are to get students to discover what the basic contents and characteristics of the subject are by introducing them to advanced study with necessary references. In connection with the third question asked I would feel it was important to offer biophysics courses to undergraduate students of natural sciences and to medical students. For students of biophysics it is a required course within which general biophysics would be an introduction to all the other special courses of biophysics.

Following on from the above it is easy to discuss the basic contents of biophysics because biophysicists have more or less the same opinion about the contents of biophysics courses, particularly since it is a problem which has been discussed over a very long period of time. During this period biophysics has matured and nowadays it is not an ancilliary

field of biology or physiology, nor is it purely molecular biophysics because it deals with the whole organism and more complicated systems. Moreover biophysics is not only an analytical science, but also an integrated science. If we agree these points, I think the following basic contents of general biophysics would be accepted by most biophysicists. I suggest the following ten chapters of biophysics: **Molecular Biophysics; Membrane Biophysics; Cellular Biophysics; Excitation Biophysics; Biophysics of sensory organs; Photobiophysics; Radiobiophysics; Irreversible process of thermodynamics; Bioinformation theory and biocybernetics and biophysics of development and evolution.** (One suggestion received was to change the last chapter to **Environmental biophysics**).

The contents listed above are only a general outline to reflect ideas. In recent years, there are two books which were published in 1983. One is 'General Biophysics' (English edition) written by Volkenstein, the other is 'Biophysics' (Ed. by Hopper et al). If necessary we could induce all the contents of the latter book into the above ten chapters. I mention these two books because the contents of the two books are similar to each other and it seems to coincide with what I have said.

To ascertain the basic contents of general biophysics is not easy, but relatively speaking it is easy. Why? When we recall the discussion of the origins of biophysics in past years it is not surprising because there have appeared some simple definitions of biophysics. For example it has been said 'Every body agrees that, whatever one is researching or teaching and calls biophysics, is biophysics'. Of course, this is an extremely simple definition.

Finally, I would like to raise a more difficult problem. What is the dominant idea or philosophy which will run through all the contents of the course or book? In simple words, the question is 'What is the philosophy or dominant ideas of biophysics'. In 1982, Professor R. Glaser had mentioned in the symposium on Education and Development of Biophysics "the basic idea of biophysics is concentration on the insight that, for all the complexity of the biological system, its basic functions are ultimately based on the laws of thermodynamics and quantum mechanics". Vokenstein had written in the preface of his book: "Here the biological phenomena realised at the supramclecular and cellular levels of structure are studied. These complicated processes occur in organisms mainly in

conditions far from thermodynamic equilibrium, so that their theoretical investigation is based on thermodynamic irreversible processes, that is, on kinetics". The ideas of these two professors are similar, it can be considered as one of philosophy of biophysics, I would like to agree these ideas, but I am not sure if it would be better to treat the contents of biophysics with the unity of matter, energy and information as a whole. I think, if we can do so, then the contents mentioned above have to be rearranged and reorganised. I think, if we can write a book in such a way, then biophysics will have its own scientific system. In that book, the chapters will be arranged systematically and logically, it would be very different from the above list. I hope all of you will give your ideas or suggestions or comments.

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THE FIRST TWO YEARS OF THE LATIN AMERICAN SCHOOL OF BIOPHYSICS SPONSORED BY IUPAB - A PROSPECTIVE VIEW

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Before turning our attention to the first two years of the Latin American School of Biophysics, a model project for regional research development sponsored by IUPAB, I believe that it is appropriate to consider its origins in order to properly appreciate its perspectives.

THE ORIGINS

It formally was in 1981 immediately after the VIIth International Biophysics Congress held in Mexico City, where I had the great honour to be chairman of the Congress organisation, that IUPAB sponsored at Oaxtepec, Morelos, the first International School of Biophysics, 'Carlos chagas'. The main objective devised for the school was to stimulate the development of experimental biophysics in the Latin American region of the American Continent at the graduate level. For that purpose one hundred and eighty graduate students and young scientists from Latin American countries and some fifty more from European and Asian countries as well as from the United States were selected to attend the School. They were exposed to discussions of prevailing strategies of experimental design and development at areas of biophysical research where significant scientific breakthroughs were occurring at the opening of the decade of the eighties.

A distinguished group of Brazilian, Argentinian, Venezuelan and Chilean scientists, collaborated with the Mexican organising group headed by Hugo Arechiqa, Alberto Darszon, Carlos Gomez, Antonion Peria, Jorqe Cerbon and Mario Ganda to make the School not only a highlight of the

Congress, but also a notable academic enterprise in its own right. In this line, it is also pertinent to mention the names of Kurt Wüthrich, Lee Peachey and Britton Chance, whose scientific vision and sparkling enthusiasm allowed the launching of the Latin American School of Biophysics inside an international framework. Mention should also be made of Mauricio Montal who not only provided strong initial impetus to the Congress and the School's academic organisation, but also contributed in many ways to their overall success.

It was, however, some years before the 1981 International Biophysics Congress, that IUPAB had planted in Mexico the first seed of the Latin American School of biophysics. In fact, it was in 1974, that at Oaxtepec Mexico, an International School of Experimental Membrane Biology consisting of nearly one hundred graduate students and young scientists from universities of Latin American countries and the United States were sponsored by the Mexican Society of Biochemistry and IUPAB to attend the International Symposium on Perspectives in Membrane Biology, organised by Carlos Gitler and myself and published by Academic Press in book form. Such an enterprise occurred at a time when the collision of experimental strategies of biochemistry and cell physiology, were delineating the present day profiles of membrane molecular biophysics. It was also, at a moment when Carlos Gitler had imposed his most definite individual and relevant scientific impact on the emergence of membrane biophysics in Mexico. The moment also coincided with Richard Keynes and Britton Chance orienting IUPAB policies to nourish the institutional process of development of biophysics in the Latin American region. This latter process was initiated by the sponsoring by IUPAB of the Perspectives in Membrane Biology meeting in 1974.

THE PRESENT

This brief academic journey initiated in Mexico, has led us to the current situation of the School whereby Professors Bernard Pullman, Joseph Tigyi and the Council of IUPAB together with the distinguished voice of Raimundo Villegas from Venezuela, committed and invigorated the interest of IUPAB in the subject, by recently considering the Latin American School of Biophysics as a special project.

From this new stimulus for the School, Professor Naul Grigera,

former President of the Argentinian Society of Geophysics, along with Ignacio Nelsing a brilliant "channel man" from Buenos Aires, organised in November 9 1986 at La Plata, Argentina, the first chapter of the present stage of life of the School (baptised in Spanish by Ignacio Reiana with trhe- initials ELAB, for Escuela Latino Americana de Biofisica) with an evaluation of the experimental techniques used in the biophysical studies of biomacromolecules. With students from different Latin American countries, the organisation of such events marked the first formal anniversary of the School. The analysis that was made at that meeting by a working group of the Socieded Argentine de Biofisica and by my participation on behalf of IUPAB, was also of great relevance for the future of the school. Along with Grigera and Reising, the group which elaborated the document of La Plata were; Alberto Bovereis, Patricio Garrahan and Alcides Rega; they took into account previous ideas made on the subject by Raimundo Villegas and Sorge Cerbon.

The La Plata working group considered that the regional formation of the Latin American Young scientists in colliding areas of research focussed between the territories of influence of biochemistry, physiology and molecular biology, can be stimulated by an "itinerant school of biophysics". The characteristics of the school to provide continuity and permanence to the ELAB project, would be the following:

1. To promote the development of biophysics in Latin America, by bringing together the most distinguished scientists in different areas of research with graduate students and young scientists from different regions of the Latin American Territory.

To consolidate the research activity of local Latin American scientific groups working in areas of molecular, cellular, applied and theoretical biophysics, through the strengthening of the graduate teaching activities at the regional level, and,

To facilitate the opening of new research lines that could interaction with those already existing.

2. ELAB could adopt the features of an itinerant school. For this purpose, two focal centres of regional academic activity could be established: one in the southern cone (Buenos Aires, Argentina) and another one in the extreme north of Latin America (Mexico City).

Participating invited scientists could stay at least one week in Mexico and travel afterwards to Argentina, where they could stay one more week. In this minimal scheme scientists would develop identical academic activities at each place.

The suggested geographical distribution could allow that the southern focal regional centre of ELAB (Buenos Aires) could host graduate students and young scientists from all contries from South America, except Colombia and Venezuela, who along with those interested from central and North American countries could have Mexico City as their focal regional centre. It was also considered that a third regional centre could be established in the future in the Venezuelan region.

3. The tentative duration of the school could be one or two weeks at each focal centre. Its main activity could be represented by lectures, conferences, workshops and seminars, provided by the itinerant invited scientists. The organisation of any other complementary regional academic activity would be the sole responsibility of the local organising group.

4. The Argentine Society of Biophysics (SAB) will be the south regional organiser, while the Mexican Society on Biochemistry will be the North Regional Organiser of the school. They will accomplish under IUPAB's umberella the bilateral work associated to the Itinerant School.

5. By creating two focal centres at both geographic extremes of the Latin American area, the activity of the school will have a multiplicative action, since the large travel displacement of a small group of distinguished scientists from north to south is associated with a much smaller displacement of the graduate students and young scientists which attend the regional activity, thus lowering travel and organising costs and fostering the attendance of a large number of participants at each regional centre.

6. The main financial support for the itinerant school may be promoted and provided by IUPAB along with IUB and ICSU. Its main commitment could be to cover travel, housing and meal expenses of professors and young scientists of ELAB.

This does not exclude obtaining funds wherever possible from

regional, national or institutional sources by the local organisers at each focal centre.

7. The subject area(s) at which the school could be directed will be established every three years, through the common and bilateral agreement of the organisers with IUPAB and IUB Executive Committees.

8. Research conference and workshop discussions may be oriented to foster the perspectives of future research in each region as well as to facilitate contact between scientific groups with similar scope and well established levels of excellence.

9. ELAB should adopt the characteristics of a permanent academic enterprise, organised every three years, if possible in parallel with the organisation of the corresponding International Congress of Biophysics of IUPAB. The trienal organisation could guarantee that during the period of formation of a graduate student in biophysics or physical biochemistry, the student could attend at least one ELAB regional meeting with the benefit of interacting with a distinguished group of scientists in the disciplines of his or her individual orientation.

PERSPECTIVES

The organisation of the second chapter of the Latin American School of Biophysics is underway in the second year of its life. The Executive Committee of the Mexican Society of Biochemistry has presented to the Executive Committee of IUPAB during the Jerusalem Congress, its willingness to join efforts with the Argentine Society of Biophysics and IUPAB, to organise the Biophysics school during 1989, in accordance with the conceptual framework and objectives envisioned for the School in the document of La Plata.

The central academic core of the itinerant school for 1989 will be focused on the subject perspectives in Membrane Biology II, that will follow the sequence Perspectives in Membrane Biology I, organised some years ago at Oxaia, Mexico, by Sergio Estrada and Carlos Gitler. The school will take place at Taxco, Guernero, Mexico and Argentina, with a difference of one week between each focal academic centre. The Mexican

organising group is headed by Sergio Estrada, Antonio Peria and Adolfo Gaian Sainz. The Argentinian group is headed by Alberto Boveris, Patricio Garraban and Ignacio Reising.

It is important to say that the first perspectives in Membrane Biology meeting succeeded very well with its intention of integrating outstanding Latin American and international scientific work, whose products were colliding and creating strong field effects of relevance, originality, quality and impact on the development of Latin American sciences at the interface and at the core of the membrane. I am sure that such a story will not only be repeated but surpassed, with the strengthening of the Latin American interaction originated at the membrane region.

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EDUCATION OF BIOPHYSICS IN CHINA

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I. HISTORICAL BACKGROUND

The establishment of the Institute of Biophysics, Academia Sinica in 1956 may serve as a milestone in the development of biophysics in China. From 1958 on, the need for biophysics in universities and colleges was soon recognised, and many departments, divisions or teaching groups were founded in a rather short period, as reflected in the rising part of Figure 1. However, in the beginning of the sixties, the difficulties of such a rapid development were soon revealed, not only because of a lack of experienced teachers, but also of an insufficient supply of sophisticated equipment, which is usually very expensive. This is the reason why we have the first peak in Figure 1. After 1962, only a few departments remained, and this situation lasted for more than ten years until 1976. From that time China was opened to the whole world, and we suddenly discovered the rapid development of modern biology, including biophysics with a big gap already formed between China and the Western world. Then came the second rise in Figure 1. This time the rise is steady, although not so steep as the previous one, having benefitted from our experience of previous difficulties.

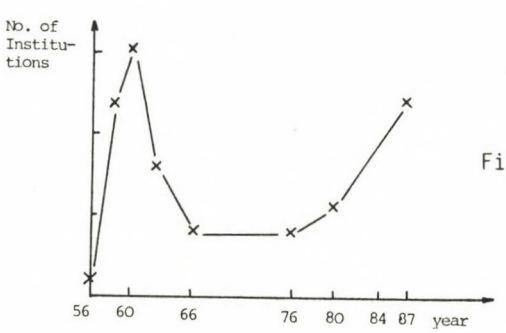

Fig. 1. Trends schematically showing the development of Biophysics institutions in China

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II. PRESENT STATUS

The present status of education of biophysics can be seen from a booklet just published named 'Biophysics in China', in which the general features of the 28 most important institutions are listed. Table 1 shows the distribution of them in various kinds of universities and institutions.

Table 1
Distribution of Biophysics Institutions

General Universities	10
Medical Universities	9
Research Institutes	4
Normal Universities	3
Agricultural Universities	2
Total	28

Among these institutions, only two universities (Fudan and Nankai) offer BS degrees, that is, they have their own students majored in biophysics. Most of them put their accent on the postgraduate training and offer MS (15 out of 28) and PhD (7) degrees.

III. PROBLEMS WE MET IN THE DEVELOPMENT OF BIOPHYSICS

There are two kinds of problems we have to solve. The first one concerns with recognition of the needs of biophysics as an independent discipline, and the second is the policy of development especially in a developing country such as in China.

1. About the necessity, two questions are always raised: What is Biophysics? Is it necessary to develop independently? Discrepancies in recognising biophysics lasted for a long time and even today no single definition will be accepted. However, no one will deny that living processes have their chemical as well as their physical bases: it is the latter one which determines the special characteristics of biophysics. So

now in China, after a long period of dispute, it is generally accepted that biophysics deals with the physical properties of living matter and the physical rules governing living processes, both from microscopic and macroscopic points of view. Although techniques are of utmost importance in developing biophysics, it is dangerous to define biophysics as the 'application of physical techniques to biology', because techniques can be used by any field in biology, which does not necessarily result in the problem being approached as biophysical one.

Objections to developing biophysics as an independent discipline usually come from scientists in closely related fields, especially physiology, biochemistry, cell biology etc. Needs for a deeper understanding of the molecular mechanisms and the physical nature of biological processes, and the needs of developing physical techniques in recent years have resulted in problem being solved in a rather natural way. Various fields are distinguished not by the problem or by the name of the biological material, such as proteins, nucleic acids, membranes, muscle contraction or vision, rather, they are distinguished by an idea, or method of approach and the nature of problem which is to be solved.

2. To establish a new borderline discipline in a developing country means to build something from without. Therefore the best way seems to be to get help from advanced countries but making endeavours by yourself are equally important. Besides the rate of development should be adjusted so as to accomodate the development of the whole country, since a developing country has so many problems to solve. The sudden rise and fall in Figure 1 evidenced the improper treatment of the above mentioned relationships.

To be more concrete, I would like to mention the following:

a. Preparation of teachers: To ask experienced physicists (chemists and mathematicians as well) and biologists to go into the field of biophysics, and prepare a new generation of students specialised in biophysics from these two approaches. However, in our cases, we had insufficient physicists at the very beginning; this situation is now changed and we are confident that this will make the development quicker. Another problem is that we needed to send students to advanced countries earlier than we were able so that we would have had more experts in this field by now.

b. Types of students prepared: As mentioned above, most institutions give postgraduate training and offer MS and PhD degrees. I think this

is correct because a solid background in physics, mathematics, chemistry and biology are equally required for all students in biology, and a broader knowledge will make it easier for students to find a job after graduation.

c. Biophysics courses. Since biophysics covers a wide range of subfields and teachers come from a variety of specialised branches, their research interests are also different. It seems therefore impossible to give a course of biophysics which meets the needs of all kinds of students. However, the fundamental principles of molecular and membrane biophysics, together with the most useful techniques (spectroscopic, diffraction and image formation etc.) in determining the structure, dynamics and functions of macromolecules and cells are generally included in a General Biophysics course. In my view, special courses at graduate level should at present stages and perhaps also in the future, be closely related to the teacher's research interest. The most common courses given now in China can be seen in Table 2.

Table 2

The most common courses of Biophysics in China

Course	No. of Institutes Giving this course
General Biophysics	14
Molecular Biophysics	14
Cell and Membrane Biophysics	10
Biophysical Techniques	15
Radiation Biophysics	6
Biocybernetics and Bio-information Theory	6
Quantum Biology	5
Neural Biophysis	4
Biorheology	4
Bioelectronics	4
Bioelectricity	3
Biothermodynamics	2
Photo-Biophysics	2

d. Research activities: Research activities of teachers are of utmost importance in improving the quality of their teaching work, not only to attract the students but also to convince them of the necessity and importance of learning biophysics for studying complex living

processes and its application in practice. Research work in China covers a variety of subfields, as illustrated in Table 3. In China, teachers are specially encouraged to do research which is intimately related to medicine, agriculture and engineering. For example the diagnosis and therapy of cancer, cardiovascular disease etc., as well as the principles of action of Chinese traditional herbs, acupuncture and so on.

Table 3

Research Fields in China

Molecular Biophysics: Crystallographic study of proteins, nucleic acids and enzymes, 3-D reconstruction of biomolecules, Energy transfer, water structure.
Cell and membrane biophysics: Membrane structure, dynamics, ion channels, permeability, energy conversion, Receptors, signal transmission and analysis, cyto-skeleton, cell cycle.
Radiation Biophysics: Primary processes, free radicals, active oxygen, bioelectro-magnetics.
Physiological Biophysics: Visual and audio-perception, muscle contraction, biomechanics, hemorheology, neurobiophysics.
Biocybernetics: Simulation and modelling of neuronetworks, systematic identification, information processing.

Biophysics education is attracting more and more medical, agricultural and engineering colleges and universities to build appropriate organisations. Of course we'll do this step by step.

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TEACHING OF BIOPHYSICS IN THE UNITED KINGDOM

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As other participants in the meeting also have emphasised, a major problem is the lack of agreement as to what fields of study should be included within the definition of 'biophysics'; in particular in the U.K. and a number of other countries, medical physics is considered to be a separate discipline and has its own societies, accreditation by those societies often being the requirement for professional employment as hospital physicists. In many other countries, however, notably Eastern Europe and South America, medical physics is thought of as part of biophysics.

Leaving medical physics aside, biophysics within the U.K. can very broadly be sub-divided into molecular biophysics and physiological biophysics, and these aspects are very largely being pursued independently. Research in physiological biophysics (for example, the processes and mechanisms involved in the origin and transmission of nerve impulses, together with the associated phenomena of ion movement in membranes) has generally been carried out within departments of physiology and these aspects are taught in physiology courses without being separately identified as biophysics.

The second area, molecular biophysics, is concerned primarily with developing an understanding of the basis of catalysis, transport processes, macromolecular assemblies, the control of gene expression, etc. in terms of 3-dimensional aspects of molecular structure and specific interaction processes that can be termed 'molecular recognition'. This area of biophysics clearly overlaps with biochemistry - an excellent illustration of this is the fact that the August 1987 issue of 'Trends in Biochemical Sciences', timed to coincide with the 9th IUPAB Congress, is a special issue on biophysics.

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Biochemists have been greatly helped in the definition of their subject by a number of outstanding undergraduate textbooks (exemplified by those of Lehninger and Stryer) that have been generally accepted as defining the bounds of their discipline. Biophysics tends to lack such an accepted 'core' undergraduate textbook and it is indeed disappointing that 'Biophysics', edited by Hoppe and others, is really too uneven in level and content to quality - there remains therefore an outstanding need for such an undergraduate text, preferably written by a single author or at most a very small number in order to ensure evenness of style.

First Degree Courses in Biophysics in the U.K.

I must first make a number of general points concerning the organisation of British universities. First, although all British universities (except one) derive their financial support almost entirely from government sources, the universities (more than 40 in number) function as independent bodies and guard their independence jealously; in particular, they are responsible for the degree schemes that they offer and are entirely responsible for the content and detailed syllabuses of their courses. Second, in most universities, the operational unit is the department and it is the department that is responsible for arranging the courses taken by undergraduate students, which will mostly comprise courses provided by staff of the department concerned, although possibly including courses offered by other departments in basic subjects. It is departments that are also generally responsible for enrolling undergraduate students and for their subsequent pastoral care. Once a student has joined a British university to study a subject (e.g. Biophysics) he will take a fairly rigidly defined combination of courses. The situation is thus considerably different from, say, the American system in which students are generally able to choose a selection of courses from what is offered by a range of departments, with many of the courses themselves being taught inter-departmentally.

First degree courses in biophysics (i.e. courses in which biophysics or a related title is used as the name of the degree the student will obtain at the end of his studies) are offered in the following four higher educational establishments in the U.K.:

University of Leeds

Molecular Biophysics (4 years)
Biophysics/Genetics
Biophysics/Microbiology
Biophysics/Zoology
Biophysics/Computational Science

University of East Anglia

Biophysics
Biophysics/Physiology

University of London, King's College

Biophysics (Biology/Physics)
Cell Biology
Molecular Biology

Portsmouth Polytechnic

Biomolecular Science

It will be seen that some of the above courses just contain the name 'biophysics', whereas others are 'combined studies' or 'joint honours' courses in biophysics and another subject. The total number of students enrolling in these courses each year is quite small, approximately 50 per annum, with about half of these attending the 'Biomolecular Science' course at Portsmouth Polytechnic, for which the entry requirements are somewhat lower than in the university courses.

The Biophysics Degree Courses at Leeds University

I shall concentrate on the course leading to the 'single honours' degree in biophysics (i.e. the course in which biophysics is the major subject throughout the student's career at the university); the 'Combined Studies' courses are made up by taking a selection of units from the single subject course, so that they comprise a smaller number of topics, but each topic being taken to the same depth as it is in the single subject course.

It is necessary first to say something about the school

curriculum in Great Britain, or rather, in England and Wales as the Scottish system is a little different. There are two principal examinations taken by students at secondary (i.e. high) schools who intend to proceed to higher education at universities or polytechnics; at age 16+, students are examined in quite a wide range of subjects, typically 8 or 9 subjects including sciences, languages, history etc.; at age 18+, they are examined in only three or four subjects and in practice most science students will only have studied three subjects in their final two years at school. This high degree of specialisation is regarded by many people as being unfortunate and there are indeed moves to broaden the school curriculum at this stage. The consequence for us at present, however, is that whereas we should like those students planning to take biophysics at university to have taken their school leaving examinations in physics, chemistry, mathematics and biology, in practice they are admitted with physics plus two of the other three subjects. We therefore divide our students into three streams according to the 'A' level subjects that they took at school and we cater for them by arranging an introductory course in whichever of the three subjects they did not take at school, as shown in the following table:

'A' levels	1st Year	2nd Year	3rd Year	4th Year
Physics	Mol.Biophys.	Mol.Biophys.		
Chemistry	Chemistry	Physics		
Maths	Intro.Biol.	Maths		
Physics	Mol.Biophys.	Mol.Biophys.	Mol.Biophys.	
Chemistry	Chemistry	Physics	Physics	Mol.Biophys.
Biology	Intro.Maths	Maths		
Physics	Mol.Biophys.	Mol.Biophys.		
Maths	Maths	Physics		
Biology	Intro.Chem.	Chemistry		

It can be seen that, in addition to the introductory courses, our students take chemistry for one year, mathematics for one year, physics for two years and biophysics in each of the four years of the course. As is indicated in the table, and will be apparent from the content of the courses, we now refer to our biophysics course as 'Molecular Biophysics' to reflect its general content.

The first two years of biophysics in Leeds can be thought of as being foundation courses and the topics in them are listed below:

Year 1 3-dimensional structures of biological macromolecules
Forces that stabilize molecular structures and interactions.
Methods of studying macromolecules in solution
Proteins, polysaccharides and nucleic acids
Cell metabolism and the coordination of metabolic processes
Structural organization in wood, muscle, membrane
Microscopy, diffraction, spectroscopy
Energy levels, transitions, dynamics of molecules and assemblies.

Year 2 Chemistry, isolation and purification of biological macromolecules
Methods of study in the solid and in solution
Symmetry in crystals, molecules and macromolecular assemblies.
Image formation: optical and electron microscopy
: X-ray diffraction
Spectrometers, spectroscopy of biomolecules

It will be seen from the above that during these two years we introduce students to the principal physical and chemical methods used to study biological macromolecules and assemblies and also introduce them to the structural nature of the molecules, the ways in which they interact in living systems and the ways in which macromolecular assemblies form.

The third and fourth years of the course consist of a number of more advanced topics, given as self-contained units, although they are clearly inter-related. Most of these units are given by members of the Biophysics Department, although we have a little help from colleagues in the Biotechnology Unit and in the Department of Medical Physics, who are responsible for the 'Medical Imaging' option.

Years 3 and 4
3-dimensional structure and function of proteins
Biological contractility (structure, biochemistry, energetics)
Molecular spectroscopy and bonding
Enzymic and chemical catalysis
Cell ultrastructure
Medical Imaging

X-ray crystallography
Quantum mechanics and advanced spectroscopy
Solution and bulk properties of macromolecules
Biological specificity and recognition
Nucleic acids, genes and viruses
States of matter
Electromagnetism and electronics

Practical work is an important element of all the courses. Each 'foundation' course in general consists of three lectures per week for 24 weeks, together with three to six hours' practical work. Students also receive tuition in tutorials, with two to six students per member of staff, either every week or in alternate weeks. In their final year, students have about 140 lectures, with each of the named units comprising either 15 lectures or 30 lectures. Final year students have some formal practical work during the first term, but the major part of their practical work is in the form of a research project, undertaken under the supervision of a member of staff. This project work is greatly enjoyed by the students and, we think, greatly beneficial to them but it is admittedly very demanding on staff time.

Career Opportunities for Biophysics Graduates

It will, I think be obvious that the course is ideally suitable for graduates wishing to enter research in biophysics either in universities or other institutions; indeed, as first degree Biophysics courses are so rare in the U.K., our graduates tend to be in great demand as research students in other universities as well as our own. Although it might appear at first sight that our course is a highly specialised one, catering ideally for research in biophysics, its overall content is in fact very broad with its coverage of the physical sciences and their applications to biology. This makes our graduates very suitable for entry to a wide range of jobs, both in science-based careers in industries such as food, agriculture, textiles, biotechnology, computing, but also for careers in which the science background is important but in which practical science is not involved, such as technical management, industrial management, technical sales, the Civil Service, etc.; a biophysics degree is also a particularly suitable entry into school-teaching.

The Changing Pattern of Undergraduate Teaching in Biophysics

Despite the excitement of contemporary biophysics, the number of students enrolling in biophysics courses and the number of institutions offering biophysics courses in the U.K. are not increasing - if anything, the reverse. This may well be due to a combination of factors. First, a number of universities have introduced courses in subjects such as biotechnology, a name that has been much in the news and is therefore thought to be fashionable. Biophysics has never caught the attention of the news media in the same way and is perhaps losing out as a consequence. Second, the demographic trend in the United Kingdom has been such that there is at present a significant decline in the number of people in the university age group, a decline that will not be reversed for another ten years or so; this means that we are attempting to increase our numbers in the face of a reducing pool of potential applicants.

Despite the disappointing trend in the number of students taking courses described as biophysics, the influence of biophysics is undoubtedly increasing. For example, there can now be few biochemistry courses in which the underlying basis of the functioning of living systems is not described in molecular terms, i.e. in terms of specific interactions between molecules arising from complementarity between three-dimensional structures. Similarly, most biochemistry courses will now contain at least some reference to the principal biophysical techniques used to investigate molecular structure, such as n.m.r. spectroscopy and X-ray crystallography, even if this is not done at a sufficiently detailed level for the students to be able to apply the methods themselves. There has thus been a substantial, even though subtle, change in the whole approach, with the ideas of biophysics becoming increasingly pervasive. Undoubtedly, there has been the same trend in courses in genetics, in physiology, and in other biological sciences.

Unfortunately, there has probably been much less of a tendency for courses in the physical sciences (physics and chemistry) to incorporate the applications of the physical sciences to the study of biological systems. There are some notable exceptions, for example in the

University of Keele and at Imperial College, London, where students specialising in physics have the opportunity to take optional biophysics courses. It is unfortunate that this tendency has not become more widespread, as undoubtedly the study of living systems provides one of the most stimulating challenges for those with a background in the physical sciences.

Biophysics Courses at Research level

In the U.K., the two principal higher degrees are the degrees of Master of Science and Doctor of Philosophy. Master of Science (M.Sc.) degrees are most commonly based on course work and are often regarded as 'conversion courses' for those who wish to change their field of study after a first degree. Apart from courses in medical physics and environmental science, M.Sc. courses in the general area of biophysics are only offered by Birkbeck College, London. Birkbeck College is a unique institution that provides part-time courses, mainly in the evening, for people who are already in employment, for example as technicians or school-teachers. Such courses are only practicable in a city such as London with a very large population to draw upon.

Ph.D. degrees in the general area of biophysics can be taken at a large number of British universities; for example, no fewer than eight universities have protein crystallography groups.

In addition to courses designed to lead to higher degrees, there is undoubtedly an incrasing demand for short courses in specialist techniques, aimed particularly at industrial scientists who need to bring their expertise up-to-date or to acquire expertise in new approaches. Universities have an important role in mounting such courses - for example my own department is running a one-week residential course on 'Molecular Graphics' on behalf of the Royal Society of Chemistry. Many such specialist courses, however, are organised on an international basis, exemplified by the series of courses and workshops sponsored by the European Molecular Biology Organization, to which may be added the programme of workshops now being planned by the newly-founded European Biophysical Societies Association.

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BIOPHYSICS EDUCATION IN EASTERN EUROPE

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Biophysics education in Eastern Europe is relatively well developed. One of the main reasons for this situation stems from the "law of uneven development", which means that those who start later usually become more up to date. Of course there are many other reasons which will be analysed later in this paper. Unfortunately, the picture given will not be complete because only data from 6 of the 8 countries were available. We had data from Czechoslovakia, Hungary, Poland, Rumania, USSR and Yugoslavia but Bulgaria and BDR did not answer our request for data. (Professor M. Markov from Bulgaria was present at the meeting and had the opportunity to present data about Bulgaria).

TABLE 1

ESTIMATED NUMBER OF BIOPHYSICISTS

		Per Cent
ON THE WORLD	25,000	100
IN THE EASTERN EUROPEAN COUNTRIES	8,350	30

Number of biophysicists. The total number of the biophysicists in the world amounts to 25,000 according to realistic estimation. (Tigyi J. (1982), Biophysics Education, Budapest pp. 1-77).

Around 30 per cent are living in the Eastern European countries, whereas about one eighth of the population of the whole world is in that area.

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It is interesting to analyse the proportion of biophysicists per million of inhabitants. The figures are summarized in Table 2. These figures are identical or rather higher than those of the Western European countries; the proportion of biophysicists in Hungary is among the highest in the world.

TABLE 2

ESTIMATED NUMBER OF BIOPHYSICISTS IN THE EASTERN EUROPEAN COUNTRIES

	NO.	NO.MILLION OF POPULATION
CZECHOSLOVAKIA	280	20
HUNGARY	600	60
POLAND	300	9
RUMANIA	150	7
USSR	5000	20
YUGOSLAVIA	250	12

A very illuminating parameter for characterising the strength of biophysics education is the number of biophysics chairs in a given area. Table 3 shows these data. So the total number of the university biophysics chairs in Eastern Europe amounts to 75 which represents 38 per cent of the biophysics chairs in the world.

TABLE 3

ESTIMATED NUMBER OF UNIVERSITY BIOPHYSICS DEPARTMENTS IN THE EASTERN EUROPEAN COUNTRIES

CZECHOSLOVAKIA	5
HUNGARY	4
POLAND	20
RUMANIA	10
USSR	30
YUGOSLAVIA	6

Besides undergraduate biophysics education all the six Eastern European countries have a well organized postgraduate educational system directed mainly by the Academies of Sciences.

The number of biophysics laboratories is also a very informative parameter about the biophysics of a given country. The Table 4 shows that figures. The size of the laboratories varies to a great extent but these figures can give at least qualitative information.

It is not an easy task to characterise the subjects of biophysical research in the Eastern European countries. However, the presentation of the main topics investigated can give us some indication of this. Tables 5-10 show the main topics of Czechoslovakia, Hungary, Poland, Rumania, USSR and Yugoslavia.

Laboratories dealing with basic biophysical research are supported by the Academies of Sciences but the medical, pharmaceutical, agricultural and nutritional firms also support a large number of research laboratories which operate in the field of applied biophysics or in very closely related areas.

TABLE 4

ESTIMATED NUMBER OF BIOPHYSICAL LABORATORIES IN THE EASTERN EUROPEAN COUNTRIES

CZECHOSLOVAKIA	20
HUNGARY	6
POLAND	35
RUMANIA	17
USSR	300
YUGOSLAVIA	12

TABLE 5

MAIN TOPICS IN BIOPHYSICAL RESEARCH IN CZECHOSLOVAKIA

1. MOLECULAR BIOPHYSICS, PROTEINS, STRUCTURE AND DYNAMICS
2. MEMBRANE BIOPHYSICS
3. TRANSPORT
4. NMR/EPR METHODS IN BIOPHYSICS
5. RADIATION BIOPHYSICS
6. THEORETICAL BIOPHYSICS

TABLE 6

MAIN TOPICS IN BIOPHYSICAL RESEARCH IN HUNGARY

1. PROTON MOVEMENT IN PROTEINS
2. PHOTOSYNTHESIS
3. STRUCTURE AND DYNAMICS OF MACROMOLECULES
4. MUSCLE-CONTRACTION
5. MEMBRANE STRUCTURE AND FUNCTION
6. RADIATION BIOPHYSICS
7. BACTERIOPHAGES
8. NEUROBIOPHYSICS
9. WATER BINDING IN BIOLOGICAL SYSTEMS

TABLE 7

MAIN TOPICS IN BIOPHYSICAL RESEARCH IN POLAND

1. MEMBRANOLOGY
2. MOLECULAR BIOPHYSICS, NUCLEIC ACIDS AND PROTEINS
3. PHOTOSYNTHESIS
4. MUSCLE BIOPHYSICS
5. THEORETICAL BIOPHYSICS

TABLE 8

MAIN TOPICS IN BIOPHYSICAL RESEARCH IN RUMANIA

1. BIOPHYSICS OF WATER AND IONS IN BIOSYSTEMS
2. MEMBRANE AND CELL BIOPHYSICS
3. NEUROBIOPHYSICS

TABLE 9

MAIN TOPICS IN BIOPHYSICAL RESEARCH IN USSR

1. MOLECULAR BIOPHYSICS
2. CELL BIOPHYSICS
3. GENERAL BIOPHYSICS
4. EXPERIMENTAL METHODS
5. AUTOMATION AND COMPUTERIZATION
6. RADIATION BIOPHYSICS

TABLE 10

MAIN TOPICS IN BIOPHYSICAL RESEARCH IN YUGOSLAVIA

1. MEMBRANE AND TRANSPORT
2. PHOTOSYNTHESIS
3. RADIATION BIOPHYSICS
4. NUCLEAR ACID REPAIR
5. WATER IN BIOLOGICAL SYSTEMS
6. EPR AND NMR IN BIOLOGY

As we mentioned above one of the main reasons for the relatively good position of biophysics in Eastern European countries is the "Law of uneven development"; conservatism and sticking to the tradition could not hinder founding new university chairs, developing educational systems and

building up the modern structure of biological research. But there are two other decisive factors which influenced the development - and of course, education in biophysics in these countries:

1. The formation of the COMECON Biophysics Cooperation System in the region and
2. The relatively large number of scientific journals with a biophysical profile.

Ad 1. The COMECON Biophysics Cooperation System was established in 1970 among the Academies of Sciences in the Socialist countries. The 6 main directions of the Cooperation can be seen in Table 11. The participating countries are: Bulgaria, Czechoslovakia, GDR, Hungary, Mongolia, Poland, Rumania, USSR and Yugoslavia. The cooperation has a Coordination Center in Moscow and the representatives (plenipotentiaries) meet every year in another country. The business meeting is combined with a scientific meeting where the most prominent results of the previous year are presented and discussed. Each of the main directions has a responsible head and a reference institute to coordinate the cooperation of the given scientific field; scientists of the main directions meet also every year in another country. The responsible authorities have a separate budget for supporting the cooperation, therefore it is very active, vivid and efficient. The scientific themes included in the COMECON cooperation enjoy priority in the national granting systems.

TABLE 11

MAIN DIRECTIONS IN THE BIOPHYSICS COOPERATION OF EASTERN EUROPEAN COUNTRIES

1. REGULATION AT MOLECULAR, CELL AND ORGAN LEVEL
2. MUSCLE CONTRACTION
3. MEMBRANE AND TRANSPORT
4. WATER IN BIOLOGICAL SYSTEMS
5. RADIATION AND ENVIRONMENTAL BIOPHYSICS
6. AUTOMATION AND COMPUTERIZATION OF BIOL. EXPERIMENTS

The Tables 12 and 13 give some information about the number of projects and the participation of different countries in the Cooperation.

TABLE 12

NO. OF RESEARCH PROJECTS IN THE FRAME OF THE BIOPHYSICS COOPERATION

DIRECTIONS	PROJECTS
1.	5
2.	4
3.	3
4.	2
5.	5
6.	4
Total	23

TABLE 13

BIOPHYSICS COOPERATION IN THE EASTERN EUROPEAN COUNTRIES

COUNTRY	NO. OF LABS
BULGARIA	17
CZECHOSLOVAKIA	34
GDR	33
HUNGARY	17
MONGOLIA	4
POLAND	15
RUMANIA	4
USSR	45
YUGOSLAVIA	12
Total	181

ad 2. Four international scientific journals are edited in the Eastern European countries. Among them the Studia Biophysica is considered as the official publication organ of the COMECON Biophysics collaboration. We should stress that the journal from the USSR is published simultaneously in English as "Biophysics" by Pergamon Press.

To sum up: we have tried to give an overview of biophysics and education of biophysics in Eastern Europe. We may state without immodesty that the situation is really good. May be it would be helpful to follow that model in building up biophysics in some developing countries.

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A clear and informative title is very important. Provide a short title (not to exceed 50 characters and spaces) to be used as a running head. Every paper must begin with a **Summary** (up to 200 words) presenting the important and pertinent facts described in the paper.

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1. Drust, D. S. and Martin, T. F. J. (1985) Biochem. Biophys. Res. Commun. 128, 531–537
2. Hoyer, S. (1980) in Biochemistry of Dementia (Roberts, P. J. ed.) pp. 252–257, J. Wiley and Sons, New York

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